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14. ABSTRACT

Surgery of the spine to fuse the vertebral bones is one of the most commonly performed operations with an estimated 350,000 Americans undergoing this surgery annually with estimated costs of $60 billion. Current procedures are highly invasive with limited success. The goal of this study is to develop a safe efficacious system for inducing spine fusion which will eliminate the need for invasive surgery. We have currently developed a cell based gene therapy system that can induce rapid bone formation at a targeted location which is independent of immune status of the model. This system relies on adenovirus transduced cells expressing bone morphogenetic protein 2 to induce bone formation leading to vertebral fusion after delivery into the paraspinous musculature. To prolong cell survival and insure cells are maintained at the target site, we have encapsulated them in a non-degradable hydrogel material. This provides additional safety by eliminating direct injection of the virus through cell delivery, and prevention of cell diffusion, through encapsulation. Here we provide preliminary data; demonstrating spine fusion using this system at 6 weeks after induction. This is the first step in demonstrating efficacy, a critical component of preclinical testing. Thus with validation of our hypothesis, this approach can now be developed as a safe and efficacious gene therapy system for spine fusion, thus circumventing the need for costly invasive surgery.

15. SUBJECT TERMS

BMP2, Spine fusion, Hydrogel, Gene Therapy, Adenovirus.

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Introduction: Surgery of the spine to fuse the vertebral bones is one of the most commonly performed operations with some 400,000 Americans undergoing this type of surgery annually in the United States. The estimated cost associated with such procedures exceeding $60 billion annually demonstrating this to be a significant problem. In the most common form, posterolateral fusion, the paraspinous musculature is stripped and the bone decorticated, resulting in significant pain, reduced stability afforded by these muscles, and disruption of the blood supply to both bone and muscle. Further, success rates for fusion range from 50-70% depending on how many levels are fused and the number and types of attendant complications. We recently demonstrated that transduced cells expressing high levels of bone morphogenetic protein 2 (BMP2) in skeletal muscle could rapidly recruit and expand endogenous cell populations to initiate all stages of endochondral bone formation, with mineralized bone forming within one week of implantation. The central hypothesis of this application is that posterolateral spine fusion can be successfully achieved with only minimally invasive percutaneous techniques and without a scaffold, by collecting cells from patient's, transducing them to express BMP2, encapsulating the cells with hydrogel material, and then delivering them to the fusion site. If added structural stability is required, the injectable hydrogel will be crosslinked *in vivo* with a small fiber-optic light source. Successful completion of this project would advance the current state of gene therapy in this field by eliminating the search for an optimal osteoprogenitor cell and scaffolding.

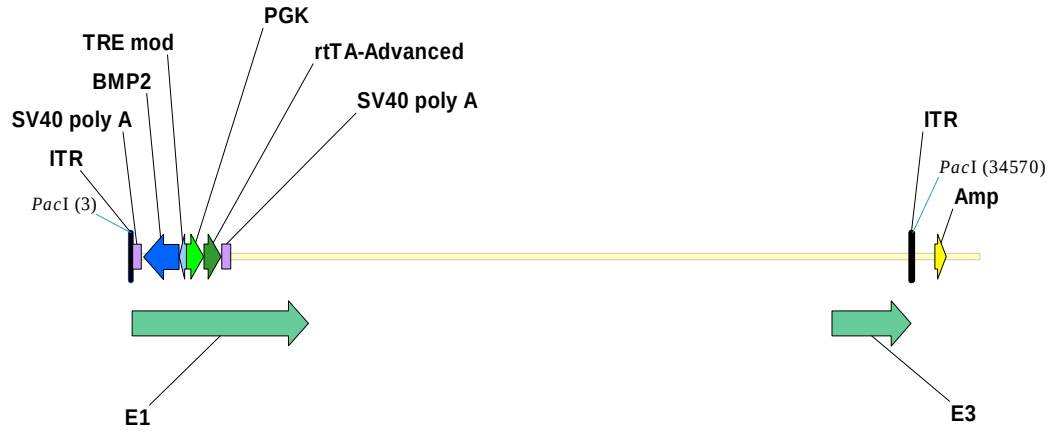
Body: The central hypothesis of this application is that posterolateral spine fusion can be successfully achieved with a novel and simple minimally invasive percutaneous technique. We propose that this can be done by transducing human fibroblasts to express an osteoinductive factor (bone morphogenetic protein 2 or BMP2), encapsulating the cells with hydrogel material, and then delivering them to the fusion site. This injectable material will be a liquid, but once in place can be crosslinked with a small fiber-optic light source. We have developed three specific tasks to accomplish our goals.

Task 1: To produce high levels of BMP2 from human mesenchymal stem cells transduced Ad5F35BMP2 adenovirus in the presence of tetracycline carrying a red luciferase reporter gene.
Viability and expression of the BMP2 will be monitored using an Ad5F35BMP2-IRESCBRluc vector that can be readily tracked using a CCD camera system, and will allow us to follow transgene expression within live animals. BMP2 expression will be regulated by tetracycline, to confirm optimal timing for bone induction. Experiments will also be performed to identify the cells responding to BMP2 and determine if this process is local or systemic. This will be accomplished using SMAD 5 "biosensor" mice in which response to BMP2 specifically leads to fluorescence emission in those cells.

a. To determine if sustained expression of BMP2 is more efficient at inducing rapid bone formation than a pulse of expression using the tetracycline regulated vectors. **(Months 0-12)**

We initiated studies to look at regulated expression of the BMP2 in the animal. First we have produced an AdE1BMP2-E3 dsRed virus that will express both dsRED and BMP2 from the same virus, but as totally different transgenes. This allows us to track cells that are expressing BMP2 and determine their viability by the live animal imaging of the dsRED (see section c). Also in this task we proposed to construct and test an adenovirus vector that was able to be regulated by tetracycline, so that we may be able to compare sustained expression to rapid pulsing of BMP2 and determine which if either produced more robust bone formation. Therefore we have constructed an adenovirus that possesses both the tetracycline activator (Tat) and the promoter regulatory element (Tre) on the same virus (Figure 1). With both tetracycline elements on the same vector, we will insure that the essential *trans* activator protein is getting into the same cell as the vector and can therefore repress or activate the transgene by tetracycline. Thus the tetracycline can effectively control all the BMP2 expression versus a subset of cells that had received both elements.

The first set contains the full length human BMP2 cDNA under the control of a tetracycline regulatory promoter element (TRE). As can be seen in figure 1, in the absence of tetracycline the BMP2 expression is turned off. As can be seen in figures 1A and B, we have two versions of this plasmid, one which has a deletion in E3 and one which has the dsRed gene expression cassette. In figure 1 we show the tet on vector, in which the BMP2 is turned on in the presences of tetracycline. We also have constructed the tet off system in which tet represses the expression of the BMP2 transgene.



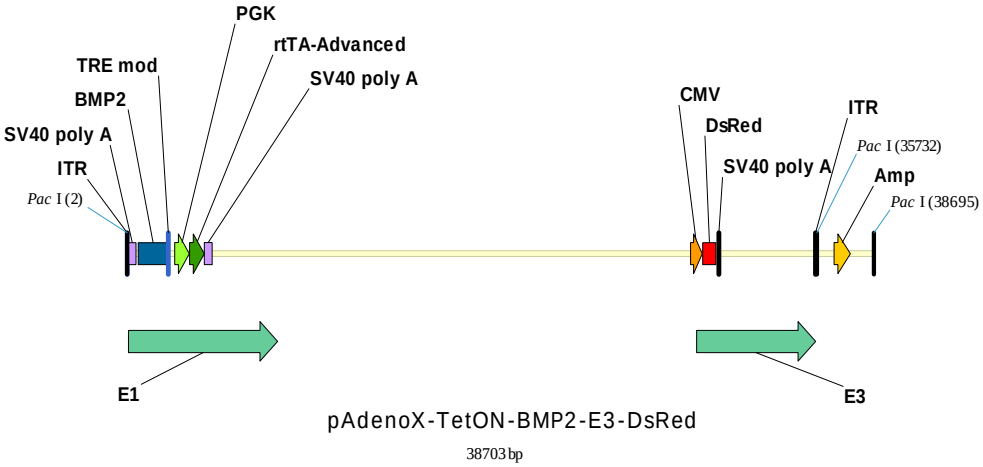


Figure 1: Schematic of the adenovirus containing the both the tetracycline regulatory element TRE and the tet activator protein rtTAT. As can be seen these elements are contained in the E1 deleted region of the adenovirus to control BMP2 expression. The second construct also has a dsRED expression cassette to track the cells expressing the BMP2 *in vivo*. The dsRED is not under tetracycline regulation, but will allow us in the absence of tetracycline to determine the efficiency of transduction, since in this case we expect to obtain little to no BMP2 production. Note we have put the two tet activator and BMP2 gene in opposite directions to avoid promoter interference. Further, we have introduced a PGK promoter driving the transactivator to avoid homologous recombination between the two promoter systems. We are the first to have success placing everything in one virus, and are disclosing this for patenting.

Figure 2 shows results using an similar AdGFP virus made using this backbone. tetracycline regulatory were In this experiment A549 cells transduce d with Adtet-GFP in the absence (A) and presence (B) of tetracycline.

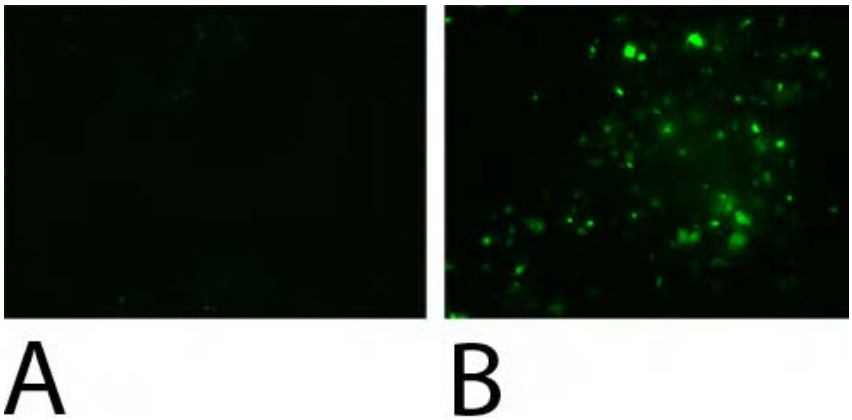


Figure 2: Fluorescent microscopy of A549 cells which have been transduced with Ad5tet-GFP in the absence (A) and presence (B) of tetracycline.

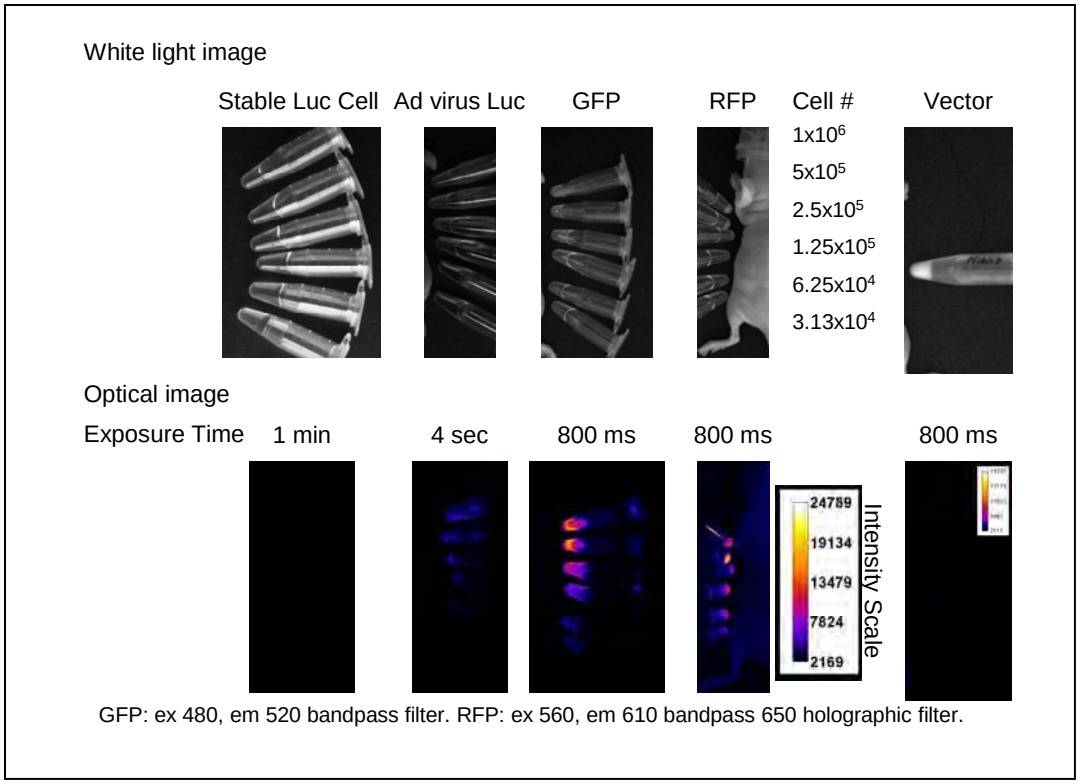
(B) of tetracycline in the media. As expected approximately 90% of the cells expressed GFP in the presence of tetracycline while only about 1% expressed the GFP in the absence. This demonstrates that the regulatory element is capable of substantial attenuation of the transgene expression and will allow us the versatility to look at pulsing BMP2 secretion versus sustained expression, to determine if there is an optimal timing for its production.

However, to implement these vectors for live animal imaging (See section C), both the BMP2 cDNA and dsRED must be under the control of tetracycline. Therefore we are also constructing these to possess a BMP2 IRES dsRED cassette. Although the internal ribosomal entry site (IRES) cassette significantly reduces the reporter gene expression level and thus allows for less sensitive imaging, two TREs elements within the same vector leads to recombination and silencing of one of the genes, making this impractical. Thus the introduction of an IRES is the only method that will allow the dsRED to directly reflect the BMP2 expression and provide feedback as to the sustained versus pulsing of BMP2 in live animal imaging (Figure 3). However we do plan to compare to static results on bone formation obtained by using the first set of vectors described in figure 1.

b. To determine if longer expression times of BMP2 from cells embedded in hydrogel and longer cellular viability lead to more rapid bone formation than the rapid but short burst of BMP2 release obtained from the cells directly injected. **(Months 9-12)**

We initiated studies to look at regulated expression of the BMP2 in the animal. We have switched from using the CBRLuc which is not very sensitive to dsRED which is easily tracked. We are currently finishing up live animal imaging of the delivery cell viability in a comparison study between hydrogel encapsulated and unencapsulated cells. **In these ongoing studies we are working towards verifying that encapsulation increases the expression time of BMP2, or alternatively masked any inflammation raised by the adenovirus transduced cells. These studies should be completed for a manuscript within this next year.**

c. To demonstrate the termination of BMP2 expression using an Ad5F35tet-BMP2-IRESCBRLuc vector in which expression can be tracked through live animal imaging. **(Months 12-24)**



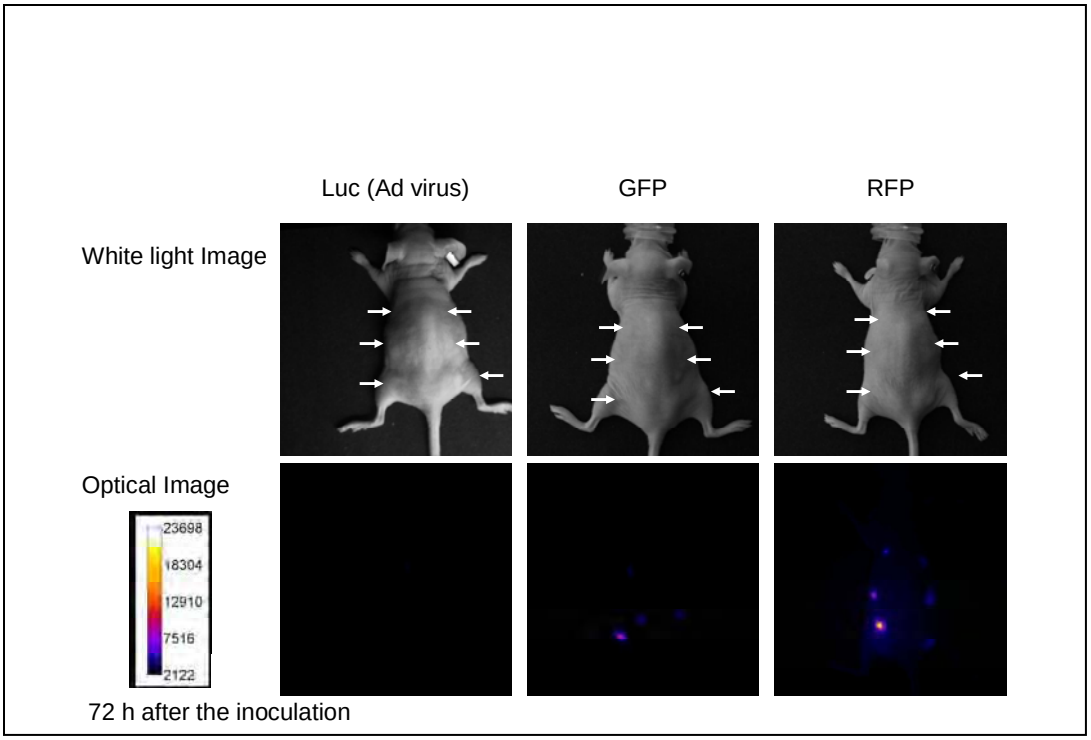


Figure 3A: Varying number of cells transduced with Ad5CBRLuc, -GFP, -DSRed, and Control "empty cassette vector" **B**: Same cells after subcutaneous injection into the mice.

We are working on developing the *in vivo* tracking system to efficiently follow the BMP2 transduced cells, and verify their location within the hydrogel materials. We are also currently developing a near infrared dye IR800 that will enter cells and bind to a peptide moiety known as halo tag (Promega Corp). We believe the near infrared will provide the greatest sensitivity. However, we are including preliminary data comparing three commonly used cell tracking modalities. In figure 3A, we injected varying numbers of cells transduced with an adenovirus (5000 vp/cell) possessing the DSRed, GFP, or click beetle red luciferase (CBRLuc) transgenes. As can be seen in figure 3, very little luciferase could be detected from the highest numbers of cells, and (fig 1B) none of the concentrations of cells were detected in the mice itself. Alternatively all concentrations of cells could readily be detected in the DSRed samples, and cell numbers ranging from 1 x 10^6 to 6 x 10^4 cells being detected in the animal after subcutaneous injection. We propose that introduction of the near infrared dye will allow us to detect as few as 100 cells. We propose to complete these experiments in the next quarter, and will then initiate the tracking experiments to determine both cell viability and exact location of the injected materials.

We have currently constructed the Ad5E1BMP2 E3dsRED vector for initial testing. We have found that this reporter to be the most sensitive, and easiest for use in the hydrogel material. We have thus initiated the experiments to track the injected cells and compare the temporal and spatial expression of dsRed *in vivo*, to that obtained from cells in the hydrogel material as described in Aim 1. One anticipated problem was the ability to see the material through the muscle tissues. Further the hydrogel may in fact shield the dsRED signal to some extent. Therefore in the initial experiment we went ahead and started with a single cell number of 5 x 10^6 cells either encapsulated in hydrogel, or directly injected into the muscles. In these experiments human MRC-5 cells were transduced with the Ad5E1BMP2E3dsRED and then either encapsulated in hydrogel, injected and photopolymerized in place, or directly injected into the hind quadriceps muscle. Due to difficulties with the animal facilities, we did not follow the same mice throughout but rather had different mice per time point. However, we are currently set up to do a second study in which we will follow the same animal for a longer period of time.

As can be seen in figure 4, the DSred expression in the cells was readily detectable in all animals. In all cases we used both hind limbs, however, as can been seen in figure 4, often the signal could often only be detected in either the dorsal or ventral view rather than both. As is the case in animal 1, both legs have robust signal only in the two different views, whereas in animal 2 the signal is only detected in the ventral view in both

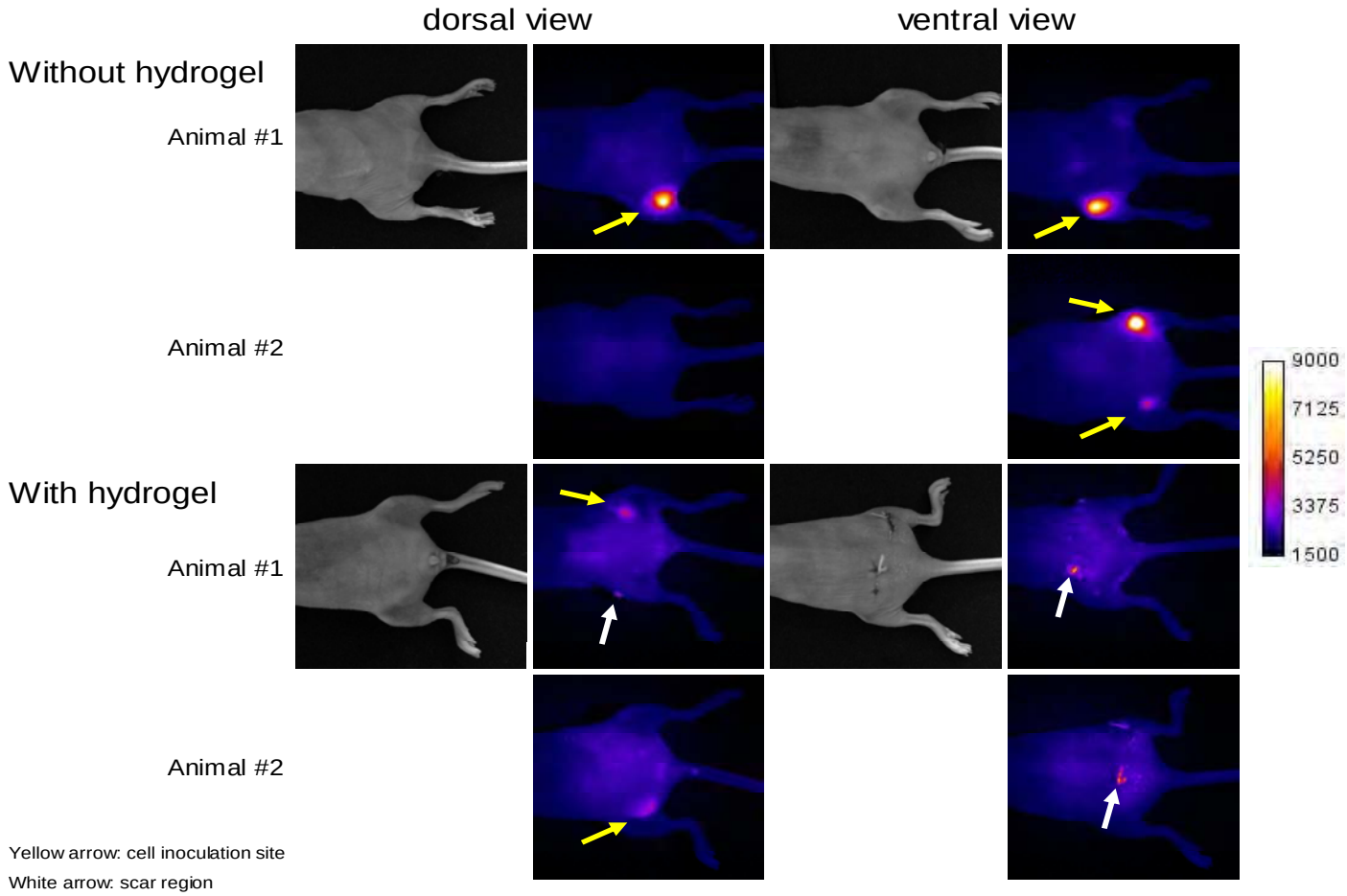


Figure 4: Live animal optical imaging of nude mice which received 5 x 10^6 MRC5 cells transduced with Ad5BMP2 and encapsulated or unencapsulated in hydrogel. In these experiments the cells were directly injected into the quardriceps muscle or fibers were separted, the hydrogel and cells injected, and then the fiberoptic light shown on the gel material to crosslink the material in place. The mice were then imaged 4 days later.

legs. Interestingly, we did not see much back ground signal from the hydrogel material, in fact the signal seemed to be somewhat shielded as expected, however, still detectable in each limb (as marked by the arrows).

We then imaged mice at a second time point, approximately 7 days after delivery of either the Ad5BMP2 transduced MRC5 cells or those encapsulated in hydrogel. As can be seen in figure 6, we still observe some signal in one of the animals that received the direct injection although it is reduced from that obtained in the

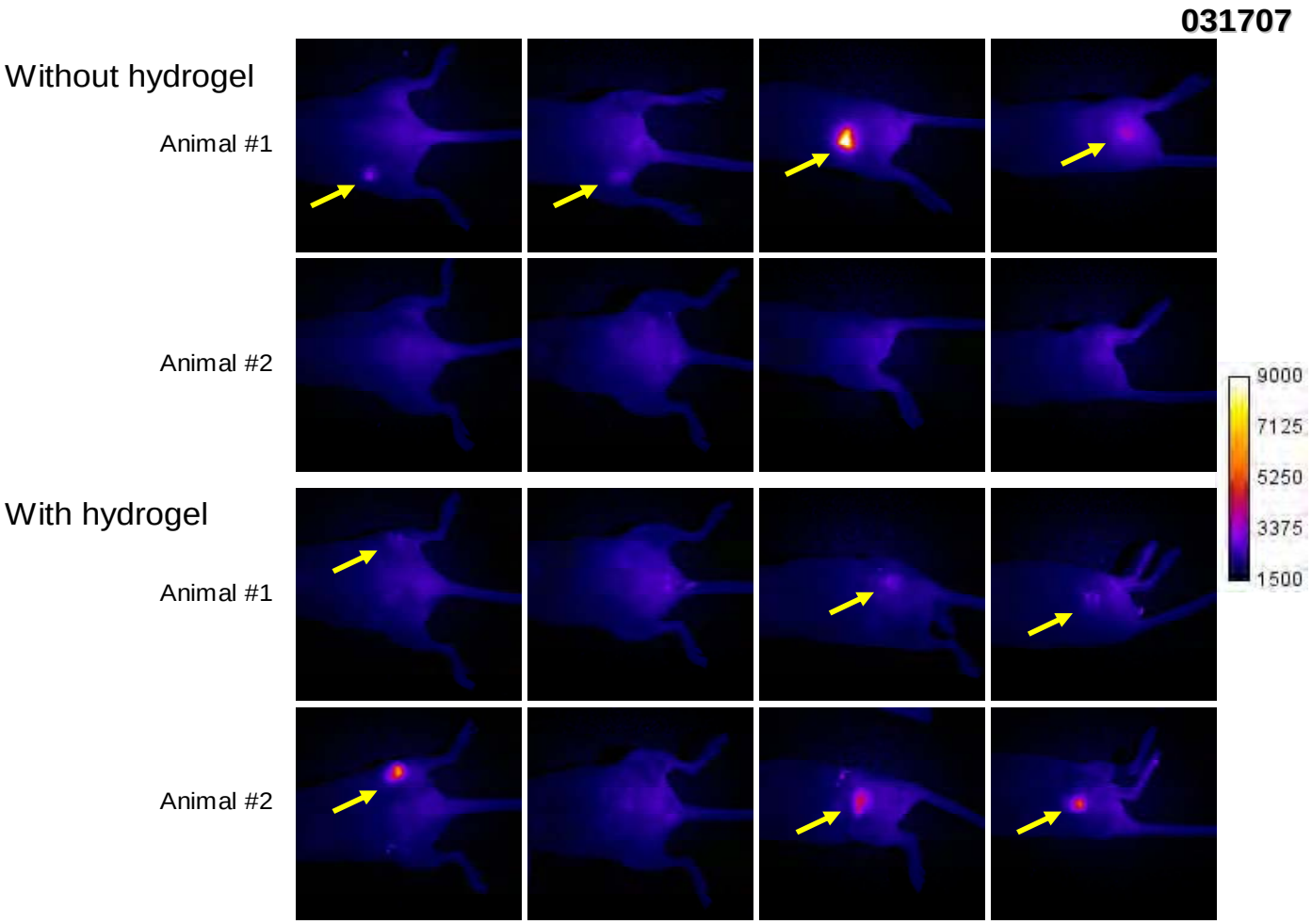


Figure 5: Live animal optical imaging of nude mice which received 5 x 10^6 MRC5 cells transduced with Ad5BMP2 and encapsulated or unencapsulated in hydrogel. In these experiments the cells were directly injected into the quardriceps muscle or fibers were separted, the hydrogel and cells injected, and then the fiberoptic light shown on the gel material to crosslink the material in place. The mice were then imaged 7 days later.

animals measured on day 4. However, the second animal directly injected had no signal in either view, suggesting that the DSRed containing cells were no longer present or viable to produce the transgene. This is consistent with our previous work demonstrating clearance of the cells by 7 days. It is intriguing to note why so much signal persists in one limb of animal 1, and we will section the tissues to determine if this is due in part to the stability of DSRed and perhaps was released when the cells were degraded or whether the cells are still present within the tissue. As expected the hydrogel encapsulated cells all yielded detectable signal and consistent in range with that observed on day 4 suggesting that the biomaterial is not only retaining the cells, but capable of prolonging the expression of the transgene.

In the above experiments the larger bead structures of the hydrogel appear to not only harm the expression of the reporter transgene, but are much lower levels of dsRED expression than what we can obtain now from the hydrogel microbeads, figure 6.

In figure 6, this is one static point from a longitudinal study currently ongoing, to verify that the microbead structures retain the cells and in turn transgene expression much longer than the direct injections. **This data is part of a manuscript which we are currently working on assembling.** We have changed the types of hydrogel structures that we have been using, from a larger bead structure to a smaller microbead structure that contains between 1-100 cells, with minimal hydrogel material encapsulating this (see task 2c for details). We have found the cells to tolerate these structures, better than in the larger structures where, the nutrient diffusion may not be as uniform. Our preliminary data suggests that cells within the interior center of the larger hydrogel bead structures die within a few days, possible as a result of this limited diffusion.

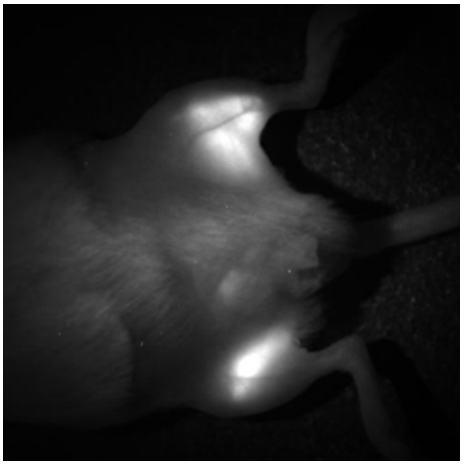


Figure 6: dsRED expression optically imaged in live animals. Cells were transduced with Ad5dsRED and then either directly injected or encapsulated with PEG-DA-hydrogel microbead structures before implantation into the hind limb

d. *To track BMP2 expression in vivo by identifying cells undergoing BMP2 signaling through confocal imaging which follows the translocation of a Smad 1 to the nucleus.* ***(Months 24-48)***

We initiated these experiments earlier than proposed since the set up of imaging this *in vivo* model is a large undertaking and will require a significant amount of time to validate. Initial experiments have been done to compare and pinpoint the changes in phosphoSmad signaling in tissues receiving the Ad5BMP2 transduced cells, as compared to those receiving cells transduced with control virus. This analysis was done in tissue sections to provide preliminary data before setting up the *in vivo* imaging.

These preliminary experiments will allow us to not only pinpoint locations within the tissues to focus, but also provide us with a time frame of signaling, thus providing incite for the in vivo studies. Figure 7, shows immunohistochemical staining for phosphoSmad using an antibody specific to the active phosphorylated form in tissue sections taken from tissues isolated four days after injection of the transduced cells.

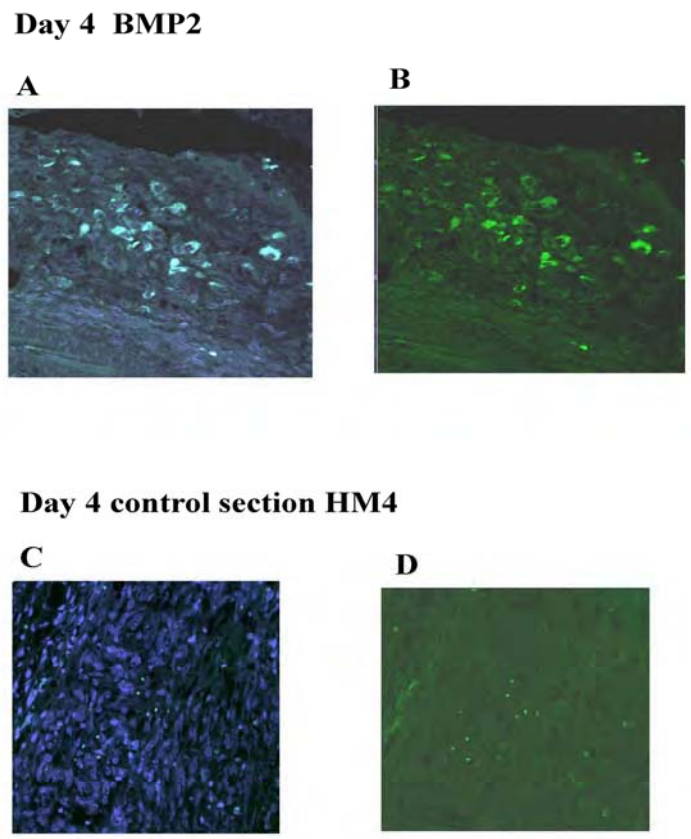


Figure 7: Immnuno-fluorescence staining on tissues isolated four days after receiving Ad5F35BMP2 transduced cells (A and B) or cells transduced with a control virus Ad4F35HM4 (C and D). Sections B and D are stained with Phospho-Smad1/5/8 antibody (1/100 dilution, Cell Signaling technology) and a secondary antibody anti-rabbit Alexa Fluor 488 conjugated. Sections A and C and stained with Phospho-Smad1/5/8 and counterstained with DAPI

These initial experiments were done in wild type (C57BL/6) mice. The phoshpoSmad mice are currently constructed and are being breed to expand there numbers, as well as further characterized as to their exact phenotype.

We next constructed a BMP signal activation indicator system for use *in vitro* and *in vivo*. We made a targeting construct to modify the Smad1 genomic locus to express the CFP:Smad1 fusion protein at endogenous levels within cells that normally express Smad1. This construct is designed to insert CFP at the N-terminal end of Smad1 by replacing the start codon in exon 2 (Figure 8). This report was tested in transient transfection experiments with COS and HeLa cells, and found to function similarly to the normal endogenous Smad1 protein, and the addition of the CFP reporter did not appear to inhibit it from translocating to the nucleus and activating gene transcription. Using homologous recombination to generate correctly targeted ES cell lines, we have generated three independent ES clones which have been injected into blastocysts for the production of chimera mice. From these chimera mice, we have now obtained germline transmission in the animals and we are currently characterizing them for the expression of CFP. We are currently expanding this colony and confirming the nuclear expression of SMAD-CFP protein.

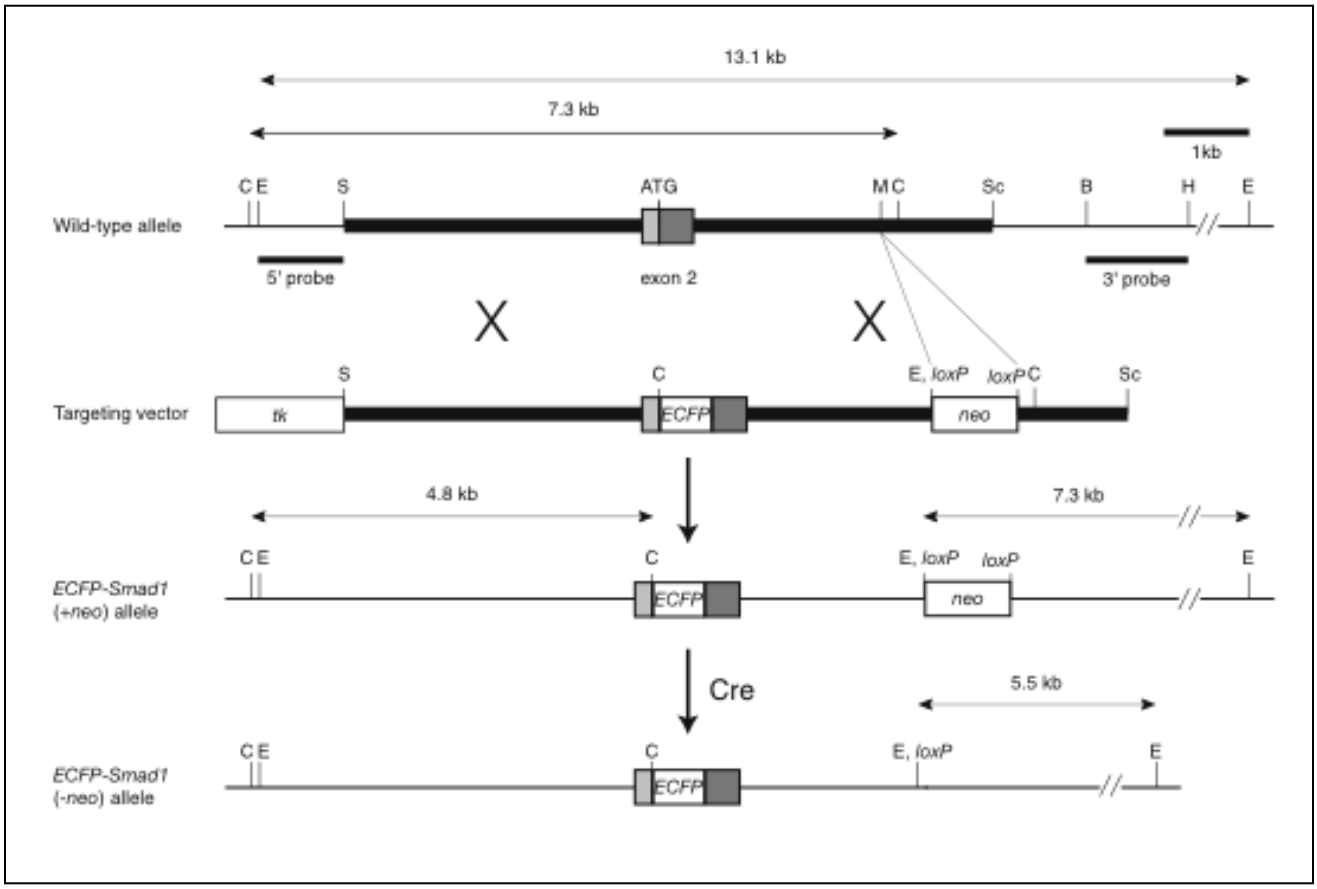


Fig. 8. *ECFP-Smad1* targeting strategy. Partial map of the mouse *Smad1* locus (top), showing the region containing exon 2 that encodes the ATG translation start site. The targeting vector is designed to create an ECFP-Smad1 fusion protein. A floxed neo expression cassette was added at an *Mlu*I (M) site in intron 2 that brings in an additional *Eco*RI (E) site. Targeted ES cell clones are identified by Southern analysis by digestion with *Cla*I (C) and hybridization using a 5' external probe and *Eco*RI digestion and a 3' external probe. The sizes of wild-type and targeted DNA fragments are indicated. Cre recombinase is used to remove the neo cassette. Thick line, region of targeting vector homology; light shaded boxes, 5'untranslated region; dark shaded boxes, *Smad1* coding region; B, *Bam*HI; H, *Hind*III; S, *Sal*I; Sc, *Sca*I; tk, HSV thymidine kinase expression cassette.

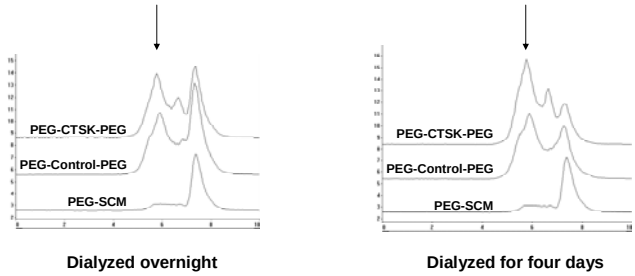
e. *Approximately 470 mice will be used in the experiments in this task. NOD\Scid 36 mice/experiment and we request three experiments, plus additional for breeding or 250 total and SMAD 1= 63 mice/experiment and we request three experiments, plus several for breeding or 220 total.*

Task 2: To design an optimal hydrogel material that will rapidly promote endochondral bone formation and be capable of removal through bone remodeling processes. In addition to BMP2 transduced cells, we propose to include peptides essential to the recruitment and migration of osteoprogenitors for bone and cartilage. Selective protease sites will also be introduced into the hydrogel to allow for osteoclast selective degradation during bone remodeling. We propose to do this by incorporation of calcium into the material and inclusion of cathepsin K protease cleavage sites into the material. Inclusion of these factors in the hydrogel will provide a mechanism for removal of the hydrogel once bone has formed by using the normal bone remodeling process.

a. Optimize and develop a hydrogel that can be specifically degraded by osteoclasts. **(Months 0-24)**
We have synthesized the peptide MGPSGPRG (Gowen et a;1999) using a 431A solid-phase peptide synthesizer (Applied Biosystems, Foster City, CA). In order to make degradable PEG, we start with PEG-DA-SMC (succinimidyl carbonate). The PEG-SMC is conjugated with our peptide in order to get PEG-PEPTIDE-PEG. So, we expect to obtain three peaks representing the completely conjugated product: PEG-PEPIDE-PEG, incompletely conjugated product: PEG-PEPTIDE and unconjugated product: PEG-SMC (Figure 9).

Synthesis of Cathepsin K degradable PEGDA hydrogel

- Cathepsin K-sensitive sequence (CTSK):
  - MGPSGPRGK
- Control sequence:
  - MPGSPGGRK
- Conjugate with 3400 Da PEG-SCM

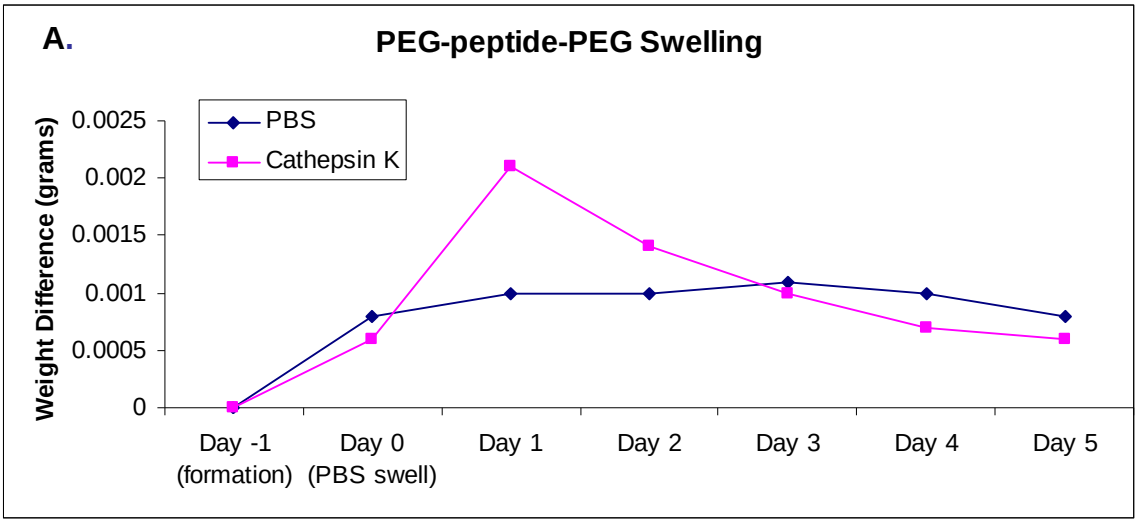


Dialyzed overnight | Dialyzed for four days

Figure 9: Results of HPLC analysis of the conjugation of peptide with the PEG-DA hydrogel.

We have run GPC tests on it and the results indicate that there is still unconjugated PEG, ie: we have PEG-Peptide or free PEG in the preparations. We have tried changing the ratio of peptide to PEG, changing the length of conjugation time, increasing the pore size of the dialysis membrane and changing the length we dialyze the conjugation products. These have led to a higher concentration of PEG-PEPTIDE-PEG as indicated by the GPC results (Figure 9). We have cathepsin K (Calbiochem; Cathepsin K, His•Tag®, Human, Recombinant, *E. coli*) and are ready to test their degradability.

The next step in developing this material was to test whether the conjugation of the cathepsin K site is enough to degrade a polymerized peptide. We placed microbeads of the hydrogel in the 25 µL cathepsin K vial (stock solution 0.2 mg/mL cathepsin K) incubated at 37°C. The PEG-peptide-PEG hydrogels (where "peptide" refers to the cathepsin K degradable peptide) were tested for swelling and degradation in PBS and cathepsin K, respectively (n=1). A swelling hydrogel typically has a logarithmic curve, like the PBS curve, where the weight of the gel increases and reaches a maximum. A degrading hydrogel will initially show an increase in weight as broken bonds allow more water in, allowing the hydrogel to swell more. Then the swollen weight will drop dramatically. Figure 10A and B shows evidence that the hydrogels are at least partly degradable. However, the results also suggest that the material cannot be completely degraded suggesting the PEG-Peptide-PEG is limiting, or that the sites are not efficiently digested. To circumvent the latter, we are resynthesizing the peptide to include additional 3 glycines on either end to lengthen the protein region and ensure that cathepsin K can reach it efficiently within its PEG backbone. Hopefully with the additional amino acids on the peptide site, any constraints the protease may have in binding for digestion of the site will be alleviated.



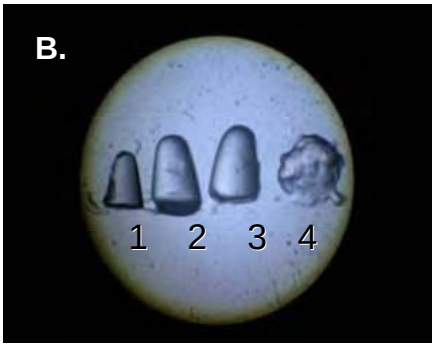


Figure 10: Swelling test of the Cathepsin K degradable peptide. **A.** Weight changes upon swelling during degradation. **B.** Hydrogels after swelling and degradation. (1) PEG-CTSK-PEG in buffer w/o proteinase K; (2) PEG-CTSK-PEG w/ 0.125 mg/ml; (3) PEG-control-PEG w/ 0.125 mg/ml; (4) PEG-CTSK-PEG w/ 0.250 mg/ml after compression.

We have been recently testing the new protease site which now has a glycine repeat sequence was designed into the peptide in order to act

as a spacer. Thus the modified peptide sequence is GGGMGPSGPWGGK, see figure 10. The W is tryptophan that we will use to track the degradation of the hydrogel by measuring tryptophan release into the media by taking Ultra-Violet/Visible (UV/VIS) Spectrophotometry absorbance measurements at 280 nm.

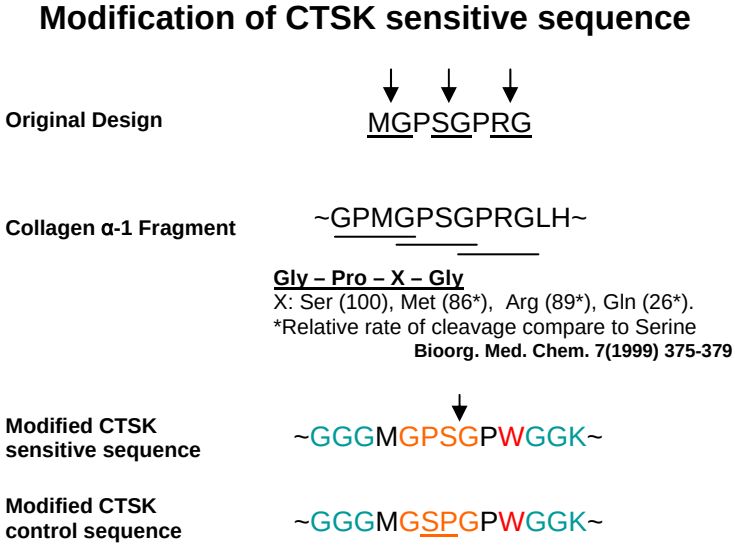


Figure 11: Schematic of the changes made in the peptide to introduce the cathepsin K protease site into the PEG-DA hydrogel.

Rather than incorporate this new peptide immediately into a PEG backbone, it was tested directly. We ran mass spectrometry on the peptide in order to see if the predicted molecular weight (1143.43) of the peptide was present. We found a peak at 1143.9, indicating success of the peptide synthesis. Next, we incorporated the peptide with activated cathepsin K and repeated the mass spectrometry. The 1143.9 peak was now absent, and present were predicted cleavage products (figure 12)

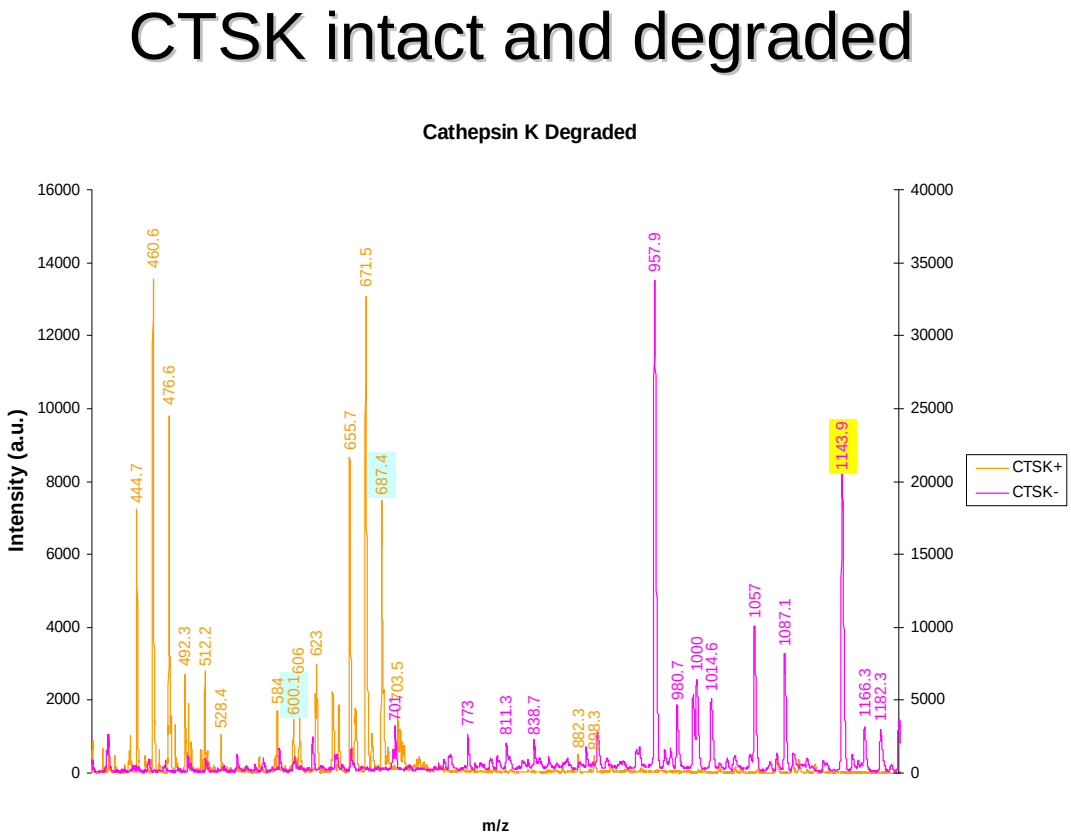


Figure 12: Mass spectrometry of the peptide consisting of the cathepsin K protease site (CTSK) before and after cleavage with the protease.

We also discovered that our control peptide had a cleavage site that we did not immediately notice until after it had been made. We will need to re-prepare the control to eliminate this site.

We proceeded with degradation tests when prepared hydrogels degraded almost immediately in proteinase K. To prevent artifact from increased enzyme concentration due to evaporation over time, we used a concentration of proteinase K that would degrade over hours, rather than days, and matched that concentration for the cathepsin K degradation tests. We were pleased to see that the tryptophan release test suggested that the hydrogels would be completely degraded by the cathepsin K similarly to when protease K was added (figure 13). This demonstrates that they had adequate incorporation of the peptide into the hydrogel and that the selective enzyme cathepsin K could cleave it.

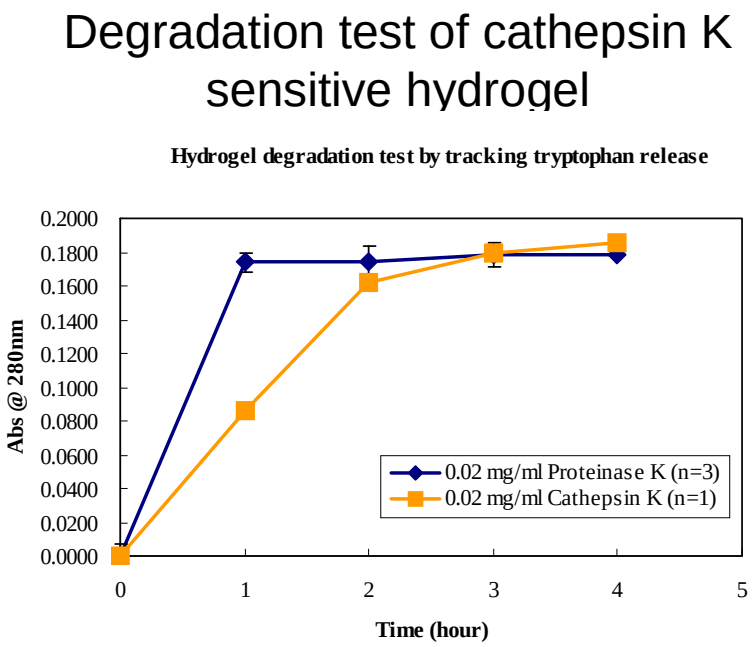


Figure 13: Trytophan release demonstrated complete degradation of the hydrogels by either the proteinase K or cathepsin K.

We next need to demonstrate the selective cleavage using enzymes that do not cleave. These studies have already been initiated. Further, we will also run concentration curves to determine the minimum amount of cathepsin K required for efficient degradation. Figure 13 shows the degradation of the hydrogel in the presence of 0.02 mg/ml and 0.1 mg/ml. As can be seen, 0.1 mg/ml can completely degrade the hydrogel similarly to more non-specific 0.2 mg/ml collagenase. Figure 14 also demonstrates the stability of the conjugated hydrogel in hanks buffered saline (HBS) without protease.

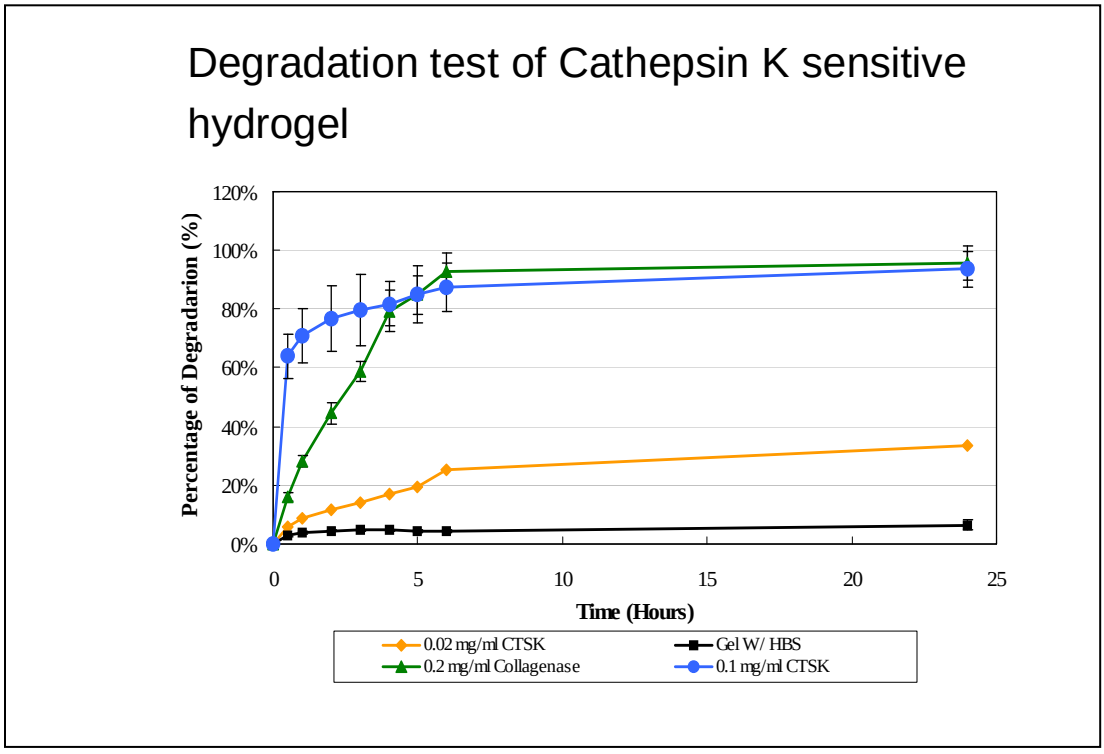


Figure 14: Degradation of hydrogel over time.

b. Test these gels *in vivo.* **(Months 36-48)**

c. Engineer cellular binding sights within the hydrogel to determine if this improves, cell viability of the transduced cells, and in turn BMP2 expression, and to tentatively enhance the migration of mesenchmyal stem cells to the sight of bone formation. **(Months 24-36)**

We have altered this aim slightly, since RGD binding sites were introduced into the hydrogel material, which did not appear to improve either the secretion of BMP2 or the MSC viability within the hydrogel. So we next opted to optimize the structure of the hydrogel's themselves. We compared the BMP2 secretion from larger hydrogel structures to the microbead structures. Figure 15 shows the significant decrease in BMP2 secretion from the larger bead structures as compared either to the direct injection of the smaller structures. The cells appear to tolerate this structure much better than the larger structures in which the cells within the center start to undergo necrosis. Development of the soft hydrogel materials for encapsulation of BMP2 transduced cells, to enhance BMP2 expression. We have been working on characterizing the type of degradable-PEG-DA hydrogel structure that would provide optimal expression, similar to the un-encapsulated monolayer. We tested several structures for their ability to secrete BMP2 into the supernatant. The assay we use to detect the BMP2 within the media is a biological activity assay (Olmsted, Blum et al. 2001) (Olmsted-Davis, Gugala et al. 2002) in which culture supernatant collected from cells transduced with AdBMP2 for 72 hours is then exposured to W20-17 cells (Thies, Bauduy et al. 1992). These cells can respond to BMP2 by up-regulating alkaline phosphatase. We have previously demonstrated that the alkaline phosphatase activity increases with BMP2 protein in the supernatant (Olmsted, Blum et al 2001) (Olmsted-Davis, Gugala et al. 2002). In figure 15, we compare the BMP2 secreted from three different types of hydrogel structures with normal monolayer cells, all of which have been transduced with AdBMP2. As can be seen in this figure, the microbeads secrete consistently more BMP2 than either the minibeads of the disks. This is the smallest of bead structure and thus has the greatest diffusion from the cells encapsulated.

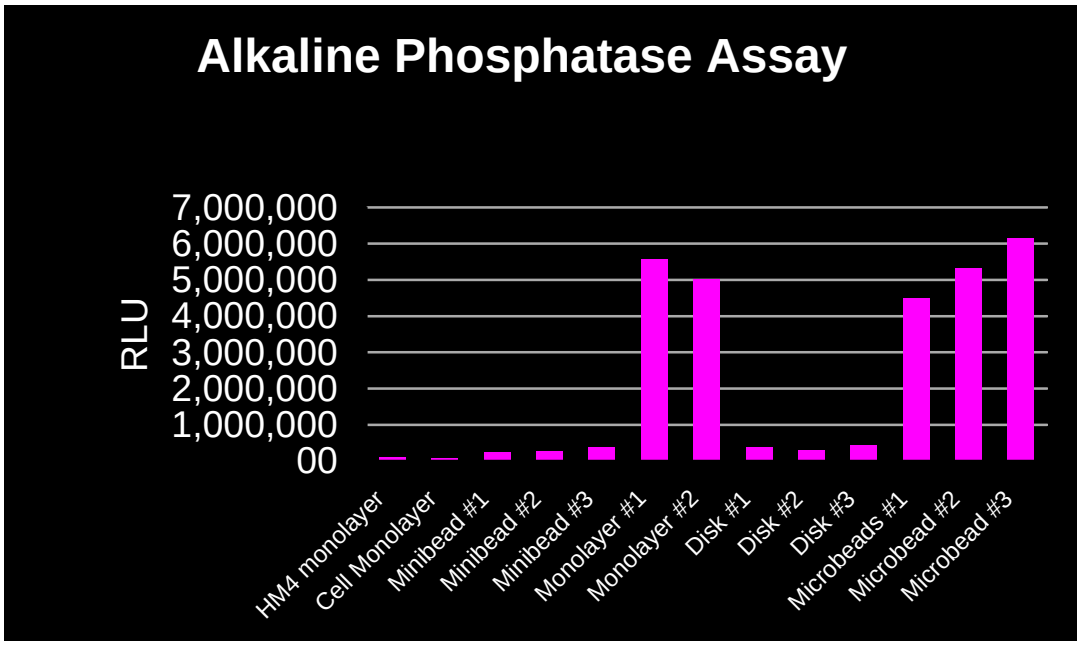
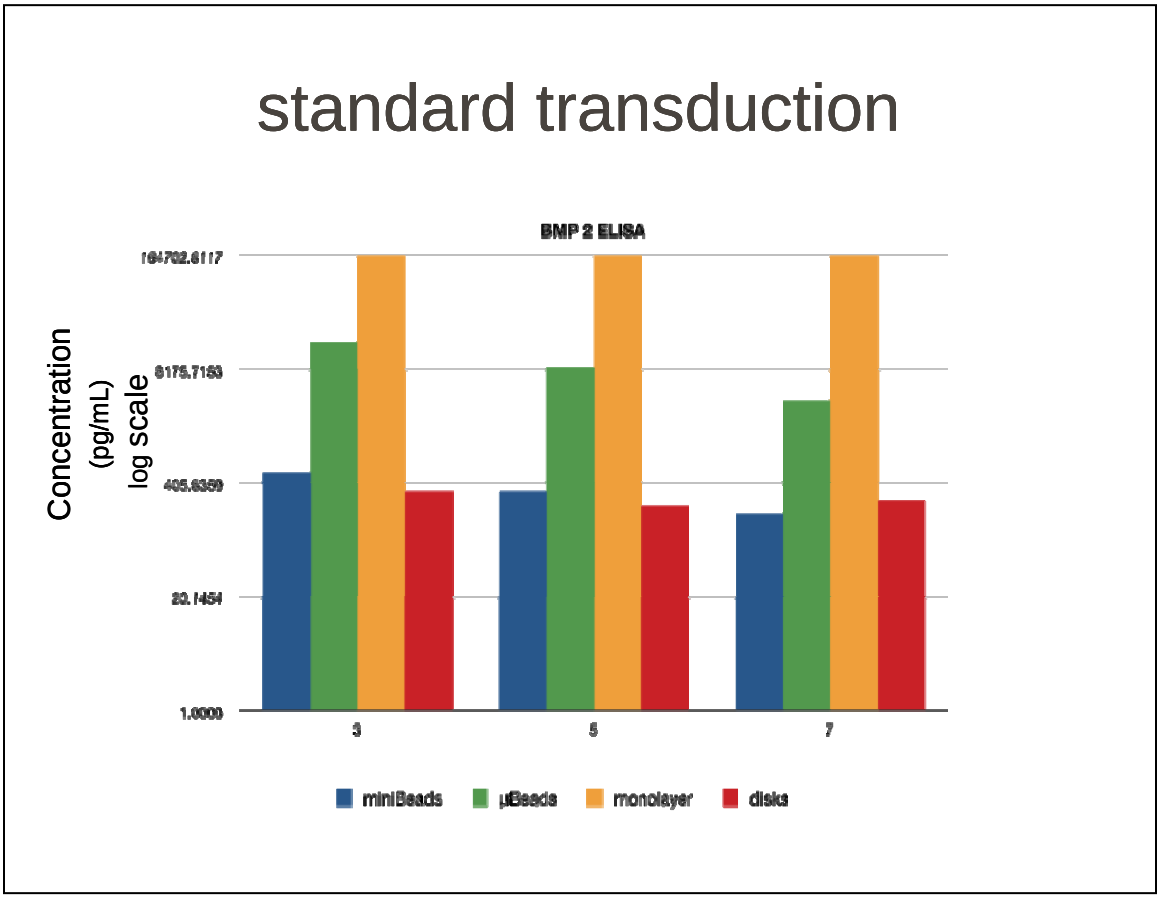


Figure 15: Alkaline phosphatase activity in W20-17 after exposure to BMP2 in culture supernatant. BMP2 activity in culture supernatant taken from cells that were encapsulated in PEG-DA hydrogel structures or plated directly after transduction with Ad5BMP2 was cultured with W20-17 cells and alkaline phosphatase activity was measured 72 hours later.



Direct measurement of the BMP2 in the culture supernatant also confirms that the hydrogel encapsulation appears to be reduced in the mini bead and disk structures as compared to the microbeads (Figure 16). The secretion of BMP2 appears to match the patterns of BMP2 activity we obtained with the culture supernatants, suggesting that the microbead structures are

Figure 16: Quantification of BMP2 in culture supernatant taken from cells transduced with AdBMP2 and encapsulated in minibead (blue), microbead (green), disks (red) and monolayers (orange). Note the scale is logarithmic, so we still observe a substantial reduction in BMP2 release from encapsulation, even with the microbeads.

efficient at maintaining the cell health and viability, for effectively producing and secreting BMP2.

We next tested the microbead structures in the heterotopic bone formation assay. Briefly, this murine assay involves delivery of BMP2 for the induction of all stages of endochondral bone formation (Fouletier-Dilling et al, 2007) and can rapidly produce mineralized bone within seven days. Here we tested the ability of the Peg-DA-hydrogel

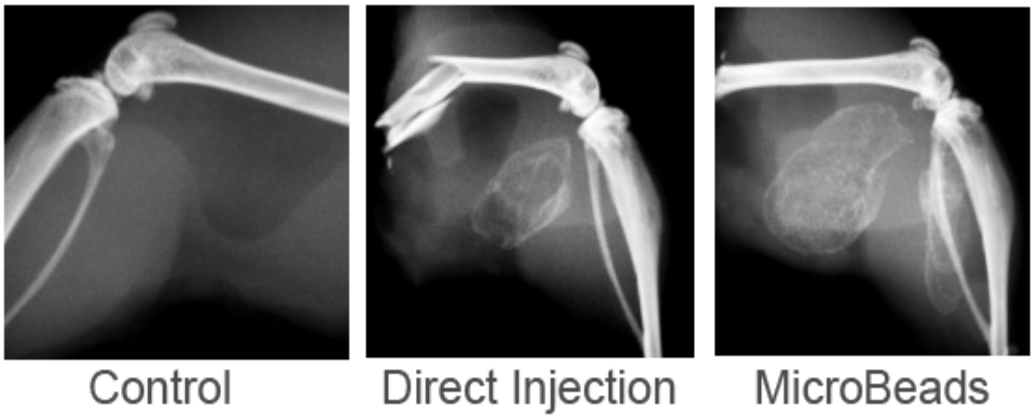


Figure 17: Radiographs of mouse hind limbs after induction of bone formation with either cells transduced with AdBMP2, or those cells encapsulated in PEG-DA hydrogel microbead structures, prior to injection. The tissues were isolated two weeks after induction of heterotopic ossification.

microbeads to form bone as compared to when we directly inject the cells. Approximately 5 x 10^6 cells were transduced with 2500 vp/cell AdBMP2 or Adempty cassette, and then 24 hours later, were either encapsulated (prior to)or directly injected into the animals. Two weeks after these injections, the tissues were isolated, and

x-rayed to visualize the heterotopic bone formation. As can be seen in figure 17, we did not observe any bone formation in animals that received cells transduced with control virus, however, we did observe extensive heterotopic ossification in both the tissues receiving a direct injection of cells transduced with AdBMP2, and in the tissues receiving those same cells encapsulated with PEG-DA hydrogel microbeads. As can be seen in figure 17, it appears that the microbeads appeared to potentially form more bone than observed in the animals receiving the cells directly, suggesting that the encapsulation into the microbead structures may aid in the production of heterotopic bone. We are currently repeating this study in a number of mice for quantification of the final bone, and are preparing a manuscript to present this material.

d. Engineer proteins that may enhance the BMP2 bone inductive response, such as VEGF-A or -D and compare with gels without additional proteins. **(Months 36-48)**

We have previously tested the degradation profile of the cathepsin K (CTSK) sensitive-hydrogel *in vitro* with proteinase K, collagenase, and cathepsin K (Figure 18). Before proceed with the animal study, we first performed a cell based *in vitro* test to demonstrate that the CTSK-sensitive hydrogel can be gradually remodeled by osteoclasts (OCs). We used the RAW264.7 monocyte cell line that has been demonstrated in the literature to undergo fusion and osteoclastogenesis in the presence of 20 ng/ml nuclear factor kB ligand (RANKL). We first differentiated the RAW264.7 cells with 20 ng/ml RANKL in standard tissue culture for 6 days, at which time we observed that multi-nuclei, osteolclast-like cells by the end of the differentiation period. Then 1.5 x 10^5 cells/cm^2 osteoclasts-like cells were seeded on 10% (W/V) hydrogel (100% CTSK-sensitive hydrogel), which contained 10mM acryl-PEG-RGDS and Alexaflour 680 labeled acryl-PEG-RGDS. The differentiated osteoclasts-like cells were then cultured on the hydrogel surface in RANKL-free medium for 24 hours before further analysis. The cells and gel were than fixed and stained with DAPI and rhodamine phalloidin after 24 hours. Images were taken by LSM-5 LIVE and then processed by Image J and OsiriX (Figure 18 and 19).

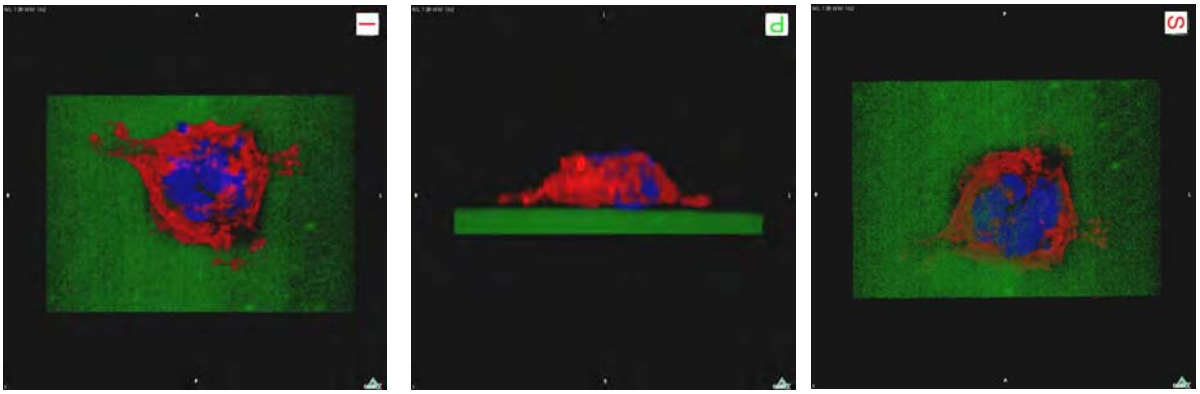


Figure 18: Fluorescence images from the (a) top, (b) side, and (c) bottom view of a single osteoclast on the hydrogel surface. The hydrogel was labeled with Alexafluor 680 fluorophore (green), which is conjugated with the acryl-PEG-RGDS and incorporated into the hydrogel by UV light induced photo-polymerization. The cells were fixed and permeabilized before stained the nuclei and F-actin by DAPI (blue) and rhodamine phalloidin (red) after cultured on the gel for 24 hours. The resorption site is located underneath the osteoclasts, which was shown by a loss of the fluorescent intensity of Alexafluor 680.

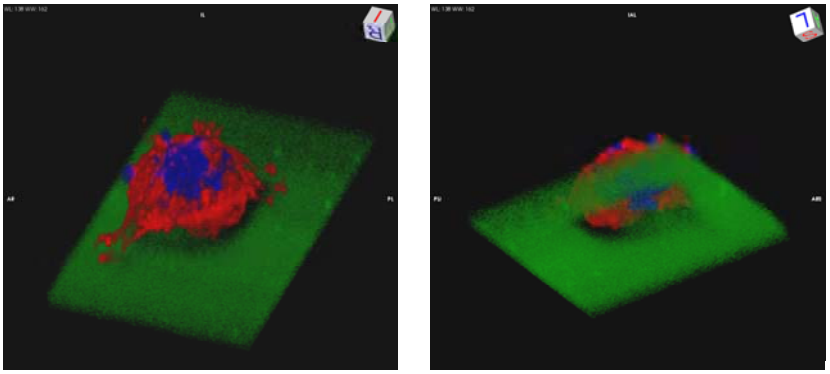


Figure 19: 3D reconstruction images of an osteoclast on the hydrogel surface. The images from the top (a) and bottom (b) view of the hydrogel were generated by Image J and OsiriX. The resorption pit on the hydrogel surface can be clearly observed from different angles.

As shown in Figure 18 and 19, the resorption site is observed underneath the osteoclast, which was shown by a loss of the fluorescent intensity of Alexafluor 680. This demonstrates that the hydrogel is being cleaved by the osteoclasts. Our next set of experiments which is ongoing is to show the inability of general fibroblasts to cleave the hydrogel. We propose to measure the area of degradation with the generalized fibroblasts as compared to the osteoclasts to confirm that we are getting selective degradation. These experiments are expected to be completed within the next 3 months, at which time we plan to publish this work.

Task 3: To achieve posterolateral spine fusion by percutaneous injection of the encapsulated Ad5F35BMP2 transduced senchymal stem cells into the paraspinous musculature of both rats and mice. Spine fusion will be assessed in both a rat and mouse model by both histological and radiological analyses over time and confirmed by both microCT and biomechanical testing. The results will also be compared to a parallel murine system which is immunocompetent. These experiments will provide essential preclinical data in two different animal models.

a. Obtain approvals through the DOD institutional review board for approval to work with the human mesenchymal stem cells. **(Months 0-12)**

This was completed within the first few months of the award.

b. Once approved we will start to utilize these cells in all hydrogel formulation experiments as described in Task 2. **(Months 12-36)**

We chose to test the system in two different murine models: immune competent and immune compromised. One major reason for testing both is that the proposed clinical system will utilize human mesenchymal stem cells transduced with an Ad5F35BMP2 vector for the production of BMP2 in vivo for spine fusion. Thus the immune compromised NOD/Scid mouse will allow us to test this system directly. However, we wished to demonstrate similar efficacy in a mouse model which is not immune compromised since presumably this system would be developed for the general population. Thus we also chose to test the system in C57BL/6 wild type mice, which require us to use a matched C57BL/6 derived cell line (MC3T3-E1). The latter system also requires the inclusion of the polyamine compound gene jammer (Fouletier-Dilling et al, 2005). However, the gene jammer is not proposed to be a component of the final gene therapy system.

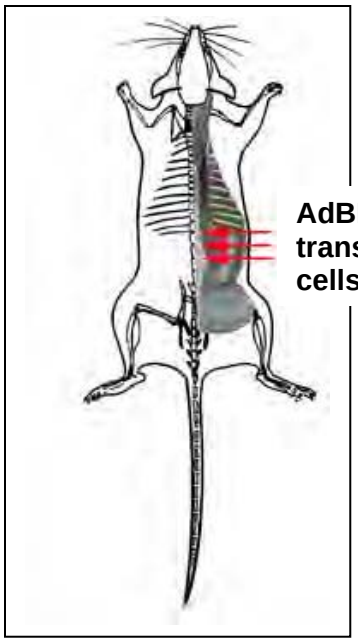


Figure 20: Schematic of the Cell Based Gene Therapy System as applied to the spine.

Therefore we next tested the ability of the system to fusion multiple vertebra in both systems. For the NOD/Scid mice human fibroblasts, were directly transduced with an E1-E3 replication defective adenovirus vector which possesses a human BMP2 cDNA, in E1 and an altered fiber gene in which Ad5 fiber has been replaced with an Ad5F35 fiber, which we refer to as Ad5F35BMP2 vector (5000 vp/cell) whereas in the second model, MC3T3-E1 cells

were transduced by addition of the gene jammer compound to the cells at time of infection with a standard E1-E3 deleted replication defective adenovirus type 5 vector possessing the same human BMP2 cDNA in E1, Ad5BMP2 virus (5000 vp/cell). Both systems routinely provide ≤90% transduction efficiency (Fouletier-Dilling et al, 2005). Next 5 x 10^6 transduced cells were injected into the parapsinous musculature of the mice (n=10 Nod/Scid and n=3 C57BL/6). To insure proper placement the material was injected under anesthesia into the mouse, along the length of the paraspinous muscle in the lumbar spine adjacent to the desired fusion point, to allow the transduced cells to track the entire muscle region (Figure 20). The mice were then euthanized at various time points and the bone was analyzed by various criteria to ensure fusion.

Radiological and microCT analysis of spine fusion:

In order to determine if heterotopic bone formation has occurred at the target site, we subjected the isolated spines, to either x-ray or microCT analysis. Figure 21A shows a microCT image of one of the NOD/Scid mice isolated two weeks after initial injection of our gene therapy system. As can be seen in this image, there is substantial heterotopic bone formation, although it appears to be less well organized and less mature, than what was observed in these animals at 6 weeks (Figure 21B). In this image one can see that the heterotopic bone formation is now well mineralized and organized along the spinal column, appears to be integrated to several vertebrae. These images are representative of what we observed in all 10 animals per time point, with a few of the two week animals appearing to be more closely resembling the 6 week animals, suggesting that a subset did in fact reach completion at 2 weeks, but not all animals tested. In no cases did we number transduced as described ses. Further, these empty cassette

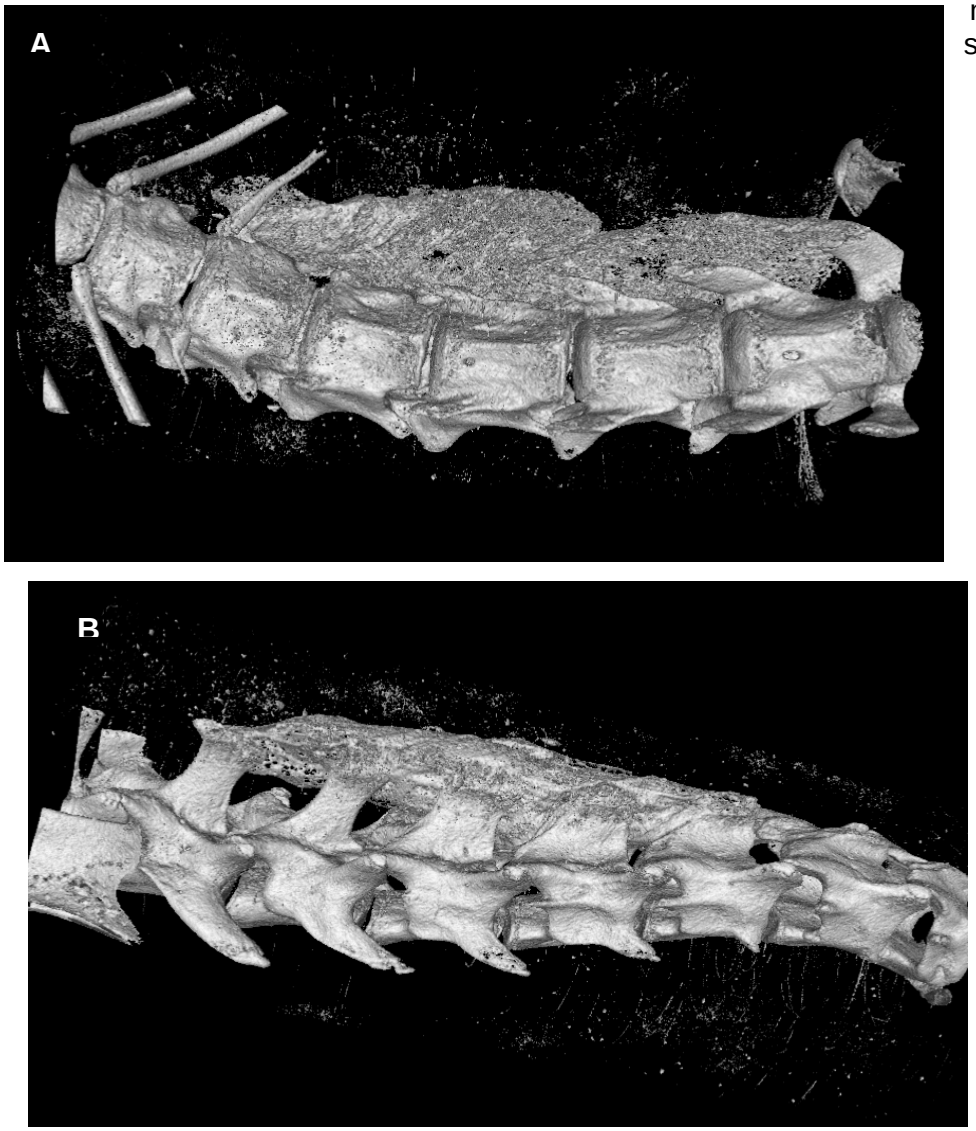


Figure 21: MicroCT anlaysis of NOD/Scid spine **(A)** two weeks and **(B)** six weeks after intramuscular injection of our cell based gene therapy system.

We observed similar findings in the immune competent mouse model. There was substantial heterotopic ossification in all animals receiving the cells transduced with AdBMP2 vectors, and no bone formation in those animals receiving the control. Figure 22 shows representative x-rays of the resulting bone formation. We are currently conducting the microCT anlaysis to determine whether we observe similar phenomena in that the two weeks appears less well organized in the majority of animals than at the 6 week stage.

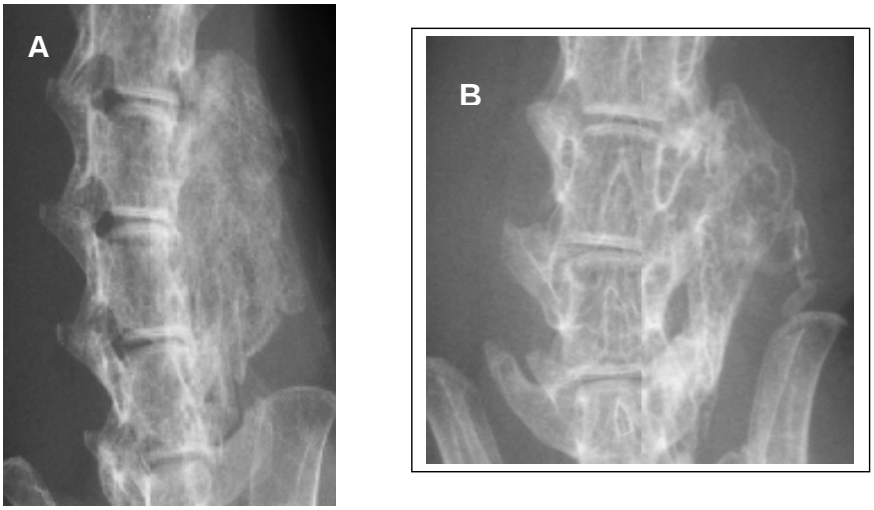


Figure 22: Radiological analysis of C57BL/6 mouse spines **(A)** two weeks and **(B)** six weeks after delivery of our cell based gene therapy system.

As can be seen in figure 22A, the spine appears to be curved towards the side which has the substantial bone formation, suggesting perhaps that the newly formed bone, is integrated or fused to several vertebra and thus in these mice which are still growing, this fusion is resulting in scoliosis. We observed similar scoliosis in several of the NOD/Scid animals as well (figure 23), again demonstrating that we have greatly constrained the spine, as well as achieved complete integration of the heterotopic bone with the skeletal bone.

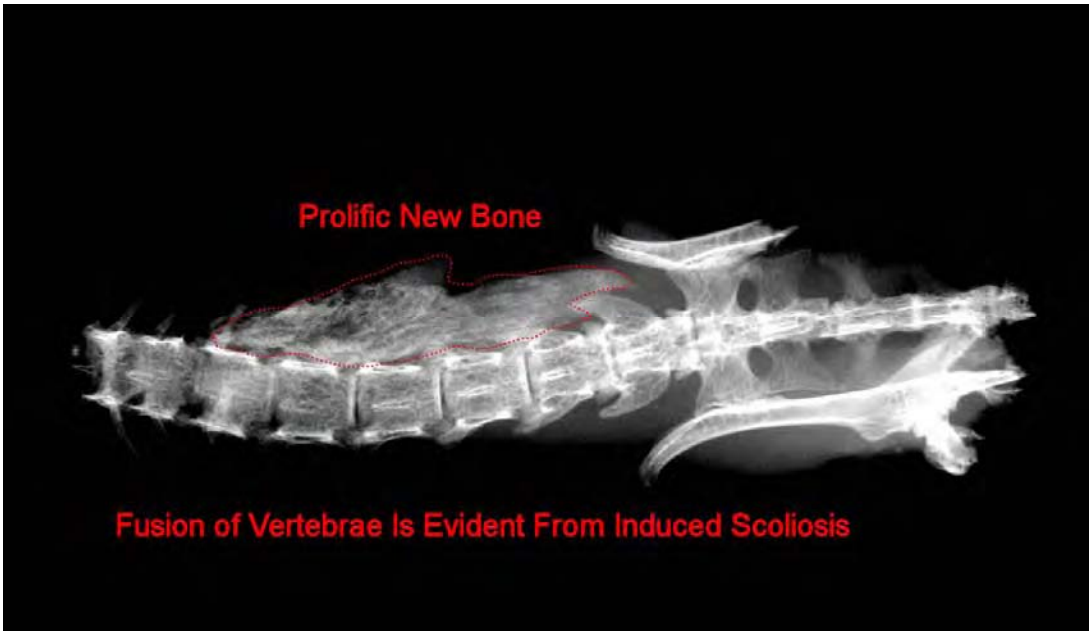


Figure 23: Spine fusion in a mouse approximately 2 weeks after injection into the paraspinous musculature of the cell based gene therapy system. Note that the mouse was still growing, and the fusion is substantial enough at this stage to cause scoliosis of the spine towards the region that is rigidly fused.

Further demonstration of the complete integration of the heterotopic “de novo” bone formation with the normal skeletal vertebrae can be seen in this cross section of the spine, generated by microCT (figure 24). As can be seen in this figure, the outer boundaries or borders of the original vertebrae have been totally remodeled and structurally integrated with the heterotopic bone.

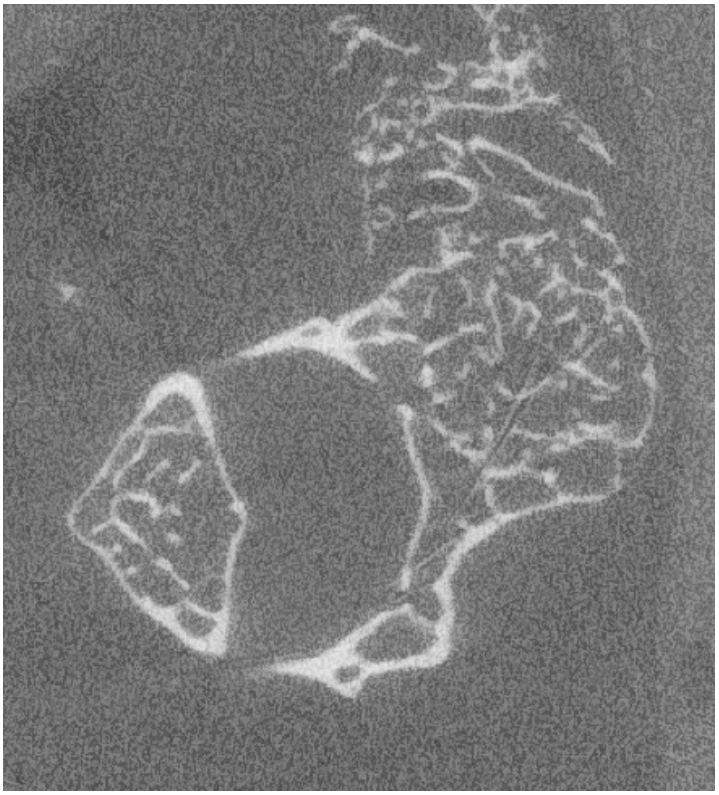


Figure 24: Cross sectional view by microCT imaging of a spine taken 6 weeks after injection of our gene therapy system into the paraspinous musculature.

Biomechanical analysis of spine fusion.
We have developed three specific tasks to accomplish our goals.

A method to reliably determine if newly mineralized tissue adjacent to the spine has formed a structurally competent bridge between vertebrae is needed to assess new spine fusion technologies in small animal models. Micro-computed tomography can be used to visualize any newly formed mineralized tissue adjacent to the spine, but it can be difficult in some cases to determine if the mineralized tissue is actually integrated with the vertebrae or if it is only overlying the vertebrae. Micro-CT exams also require expensive equipment and long-scan and post-processing times. To provide a simple and rapid method to directly test whether a fusion was mechanically successful, a mechanical device was developed that creates controlled flexion and extension in a mouse spine, so that spine fusions could be assessed using the same computer-assisted methods that are now widely used to assess spine fusions in human patients. This device was validated in a mouse spine fusion model. Utilization of the device involves embedding the spine and surrounding tissues, after removal from the body, in mold using rapid setting alginate. The embedded spine is then flexed using the device and a micro-radiograph is obtained with a digital Faxitron system. The embedded spine is then placed in extension and a second radiograph is obtained. These two radiographs are then imported into a workstation and analyzed using previously validated computer-assisted technology (Zhao KD, *et al.*2005). The software allows intervertebral motion to be accurately quantified. A successful spine fusion is intended to stop any significant motion between vertebrae. In the human spine, intervertebral motion under 1.5 degrees at any level is considered to be reliable evidence of a solid spine fusion (Hipp JA et al, 2008) using this image processing technology.

Table 1:

Group	Time	N	%Spines with 1 Fused Level	Spines with > 1 fused level
Control	2 wks	0	0	0
Control	6 wks	9	0	0
BMP	2 wks	9	44.4	22.2
BMP	6 wks	10	90	50

To test whether the tentative fusions were actually capable of reducing motion within the spine, we set up some experiments to look at flexion/tension under bending at specific angles. Briefly, three groups of NOD/Scid mice were given an injection of MRC5 cells transduced with 1) Ad5F35BMP2 (2 week analysis), 2) Ad5F35BMP2 (6 weeks), and 3) Ad5F35empty cassette all at 2500 vp/cell. The cells were transplanted into the paraspinous musculature of through direct injection. Group 1 animals were isolated at 2

weeks, while groups 2 and 3 were harvested at 6 weeks after initial induction.

Next we analyzed the spines from all groups. In the case of the control, and 2 week BMP2, we did not find any statistical difference from the normal group. Although at 2 weeks we observed heterotopic bone by x-ray and microCT, this bone had not adequately fused to the vertebral bone, and thus did not shown significant differences form the normal spines. However, results from analysis of the group isolated 6 weeks after delivery of the BMP2 expressing cells, showed a significant drop in the disc angle and in turn flexion of the spine. The reduction in flexion is consistent with what has been demonstrated in human spines as a standard for clinical fusion. Figure 8 shows the results of a representative sample from this group. The results of this analysis on n=10 spines are shown in table 1. As can be seen in Table 1, approximately 44% of the spines had significant reduction in flexibility in the spine, or fusion, at two weeks, and at 6 weeks approximately 90% had one level of fusion or greater.

Again this is from a simple injection of the material into the paraspinous musculature. We are currently testing samples from 4 weeks to determine if the fusion has occurred early than the 6 week time point as well as setting up experiments to test the microbead structures in the spine.

Gross anatomical confirmations:

To verify the amount of intervertebral motion that exists in normal mouse spines using this test method, 9 NOD/SCID mouse spines that had not been exposed to any intervention were tested. Intervertebral motion averaged 5 degrees between each pair of vertebrae in the normal mouse spine (See section task 3d). As a gold-standard to determine whether the spines were or were not fused, the mouse spines were immersed in bleach for approximately one hour after the mechanical tests were completed. This process removes all soft tissues from around the vertebral bodies. A nylon string is threaded up the spinal canal midway through this process so that the relative position of vertebrae is maintained. All vertebrae that are not fused together fall-apart during this process, and all vertebrae that are fused together remain attached (Figure 25). The average intervertebral motion at those levels confirmed to be fused after the bleaching process was significantly ($P<0.0001$) less than at levels where vertebrae separated after bleaching.

Figure 25: Results of analysis of the disc angle after bending at110, and 150 degree angles in tissues isolated from mice 6 weeks after induction with BMP2. The disc analyzed is listed as the disc between the two lettered vertebras. The disc which provided the most significance in limiting the disc angle is highlighted by the orange box. This sample is representative of the group of fused spines.

c. Once the gels have been modified to offer optimal properties for bone formation and removal, we will test these in a murine model of spine fusion. Since we have substantial knowledge of the mouse model, we will initially start to collect data with this system. We will demonstrate the ability to induce spine fusion in the presence of tetracycline. **(Months 24-40)**

We most likely will not attempt these, since we have an alternative method for looking at gene expression that may be more effective at removing the cells, and transgene expression. This relies on the caspase gene which can induce apoptosis in cells when provided an activator compound that can readily pass through the PEG-DA hydrogel. See next section.

d. Analyze the modified injectable hydrogel for optimal volume, *in vivo* crosslinking, design, selective degradation, and inflammatory reaction using both live animal imaging and histology. **(Months 24-48)**

We will no longer be crosslinking *in vivo* since we are actually attempting to perform the specific microbead structures for optimal cell viability and BMP2 secretion. These structures are actually injectable, and thus will be utilized similarly to the direct injection of the cells. Preliminary data suggests that these structures will provide for even more rapid and reliable fusion of the spine. In this next phase of the grant, we will be encapsulating human mesenchymal stem cells that possess a caspase gene, which will provide a method for timed cell removal. In this case we will add the activator of caspase to send the BMP2 transduced cells through apoptosis, paralleling the timing of remodeling of the PEG-DA hydrogel microbead structures. Thus we will ensure that we destroy the cells within the PEG-DA hydrogel microbead structures, prior to potential degradation of the polymer. These studies with the human mesenchymal stem cells will also be tested using the dsRED reporter so that we can track their viability in the presence of the drug, as well as determine the optimal timing of BMP2 secretion for bone formation. This is actually the final phase of the proposed set of studies, and should allow us to arrive at a final product for testing.

e. All fusions will be tested both biomechanically as well as radiologically using microCT to confirm the fusion. **(Months 40-48)**

A method to reliably determine if newly mineralized tissue adjacent to the spine has formed a structurally competent bridge between vertebrae is needed to assess new spine fusion technologies in small animal models. Micro-computed tomography can be used to visualize any newly formed mineralized tissue adjacent to the spine, but it can be difficult in some cases to determine if the mineralized tissue is actually integrated with the vertebrae or if it is only overlying the vertebrae. Micro-CT exams also require expensive equipment and long-scan and post-processing times. To provide a simple and rapid method to directly test whether a fusion was mechanically successful, a mechanical device was developed that creates controlled

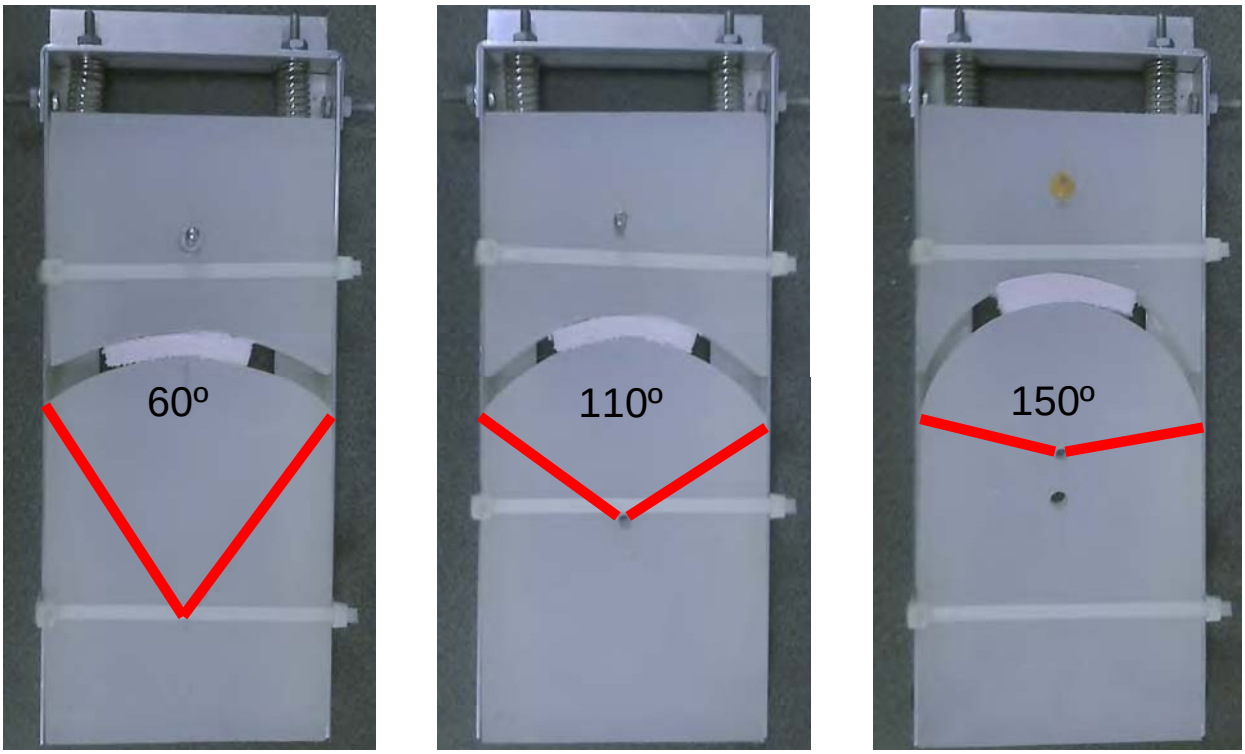


Figure 26: Angles and apparatus to flex and extend the spines after fusion, to determine if the degree of motion has been restricted in the murine model, to a similar extent to what would need to be achieved to reduce pain in a human clinical setting.

flexion and extension in a mouse spine, so that spine fusions could be assessed using the same computer-assisted methods that are now widely used to assess spine fusions in human patients. This device was validated in a mouse spine fusion model. Utilization of the device involves embedding the spine and surrounding tissues, after removal from the body, in mold using rapid setting alginate. The embedded spine is then flexed using the device and a micro-radiograph is obtained with a digital Faxitron system. The embedded spine is then placed in extension and a second radiograph is obtained. These two radiographs are then imported into a workstation and analyzed using previously validated computer-assisted technology (Zhao KD, *et al*.2005). The software allows intervertebral motion to be accurately quantified. A successful spine fusion is intended to stop any significant motion between vertebrae. In the human spine, intervertebral motion under 1.5 degrees at any level is considered to be reliable evidence of a solid spine fusion (Hipp JA et al, 2008) using this image processing technology.

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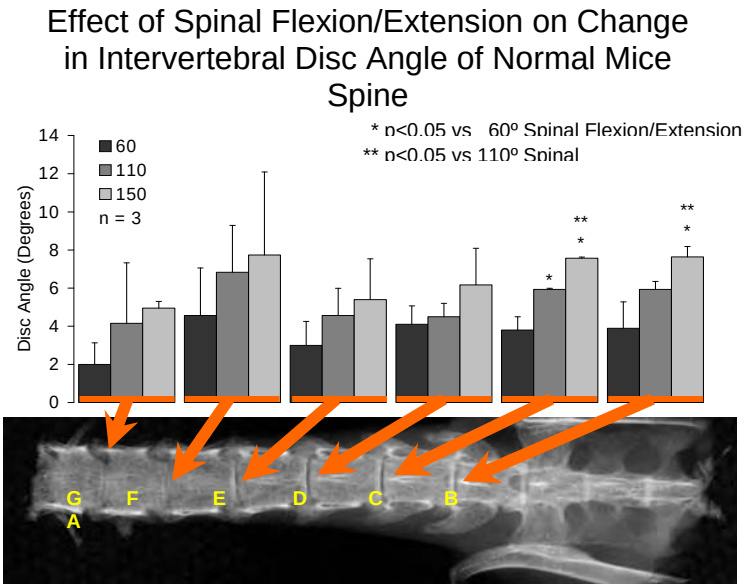

Figure 27: Results of analysis of the disc angle after bending at 60,110, and 150 degree angles. Each column of numbers represents the corresponding disc (arrow). The results suggest more variability in discs on the end of the animal, where the spine has been cut, versus internal or adjacent to the pelvis which remains on these tissues.

To determine what to expect for normal spine flexibility in these mice, we subjected a group which had received no treatment to this analysis. As can be seen in figure 27, the values obtained from subjecting the mice to 60°, 110°, and 150° angle varied significantly at the ends of the spine and most likely due to the isolation, and the fact that the spine had been cut. Discs within the center and lower spine showed significance between 60 and 110 degree shifting with little or no variability, and allowed us to focus on this region for analyzing our fusions.

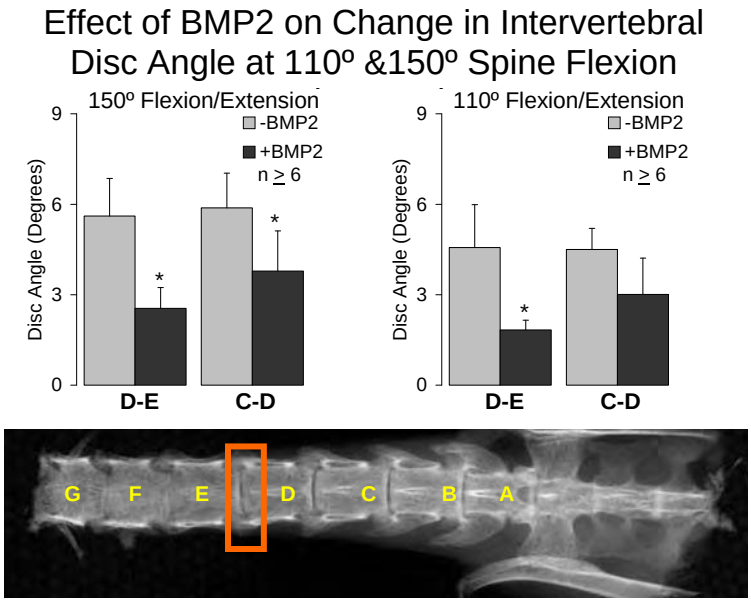

Figure 28: Results of analysis of the disc angle after bending at110, and 150 degree angles in tissues isolated from mice 6 weeks after induction with BMP2. The disc analyzed is listed as the disc between the two lettered vertebras. The disc which provided the most significance in limiting the disc angle is highlighted by the orange box. This sample is representative of the group of fused spines.

We are currently assembling these results into a manuscript.

f. Once we have established methodology that leads to reproducible fusion we will further test this in a rat model of spine fusion using athymic rat's spine fusion using athymic rats. We will then compare and confirm that these results are similar to those obtained in immuno-competent mice. **(Months 40-48)**

g. To initiate toxicology studies, as potentially outlined in a pre-IND meeting with the FDA. **(Months 24-48)**

Key Research Accomplishments

- We have developed a method for monitoring BMP2 expression in live animals through the use of dsRED. This optical imaging modality appears to be specific and significantly more sensitive than either lucerifase imaging or GFP detection. We have initiated studies to look at the regulated BMP2 carrying a dsRED gene and compare bone formation in the presence of long term versus short term BMP2 expression.
- We have developed a formulation of hydrogel that provides for sufficient BMP2 expression to induce *in vivo* bone formation. We have shown that these novel microbead structures efficiently secrete functional BMP2 and can produce heterotopic bone in the mouse hindlimb. The cells encapsulated in these structures survive a significantly longer time period than their directly injected counterparts, and they make significantly more bone than any of the other PEG-DA hydrogel structures tested. These are easily injected and appear to reside at the injected location.
- We have been working on introducing degradation sites within this material that would allow it to be remodeled by osteoclasts similar to normal skeletal bone. We have synthesized the peptide sites and introduced them into the PEG-DA strands and succeeded in getting normal crosslinking of the material. We have then degraded the hydrogel completely in the presence of either a general protease (proteinase K) or a specific proteinase (cathespin K) but not with other selective proteinases. We are currently testing the hydrogel materials in the presence of cells, either a non-specific fibroblasts (osteoblsts, and skin fibroblasts) or osteoclasts to demonstrate again the selectively degradable nature of the material. We will then seed the hydrogels with BMP2 transduced cells, and further test these for BMP2 expression as well as *in vivo* animal testing and selectable degradation.
- In these studies we set up a large number of animals for biomechanical testing to develop better criteria for demonstrating true fusion of two or more vertebra. The first groups used for this experimental design for direct injected cells; however, we are currently now testing fusions in animals receiving the hydrogel encapsulated cells. We have developed a biomechanical testing strategy to rapidly

demonstrate true spine fusion with the vertebra in our rodent and larger animal models. This is a critical component to characterizing the biomaterial, since on many occasions' microCT or x-ray analysis can appear as a true fusion, however, upon bending the heterotopic bone will break at the fusion site rather than constrain the spine, as a result of poor fusion, and remodeling with the normal skeletal bone.

- We have previously demonstrated similar temporal and rate of bone formation in comparison studies between C57BL/6 (immune-competent) mice and the NOD/Scid (immune incompetent) mouse model. We have completed studies in the spine, to obtain fusion in both types of mice (wild type and NOD/Scid). Our data shows rapid spine fusion in mice that received either direct injection of the AdBMP2 transduced cells into the paraspinous musculature regardless of the status of the immune system. We have demonstrated fusion of two or more vertebra by direct examination of the skeleton, as well as by restriction of vertebra motion (flexion-extension) using a novel biomechanical methodology. Thus we have confirmed that we can use de novo heterotopic ossification to fuse the spine rapidly (within 2-4 weeks) after a single injection into the paraspinous muscles of the animal.
- We are currently patenting this methodology

Reportable Outcomes:
Abstracts:
Oral presentation:
Ronke M. Olabisi, Chi-Wei Hsu, **Elizabeth A. Olmsted-Davis**, Jennifer L. West. Cathepsin-K Degradable Poly(ethylene) Glycol Hydrogels for Bone Formation. Federation of South-Western Bioengineering Annual Meeting, 2009, San Antonio TX (Oral Presentation).

Poster presentation:
Smith, T J; Gandy, J C; **Olmsted-Davis, E A**; Davis, A R; Sordillo LM; Zachos TA Efficient *In Vitro* Adenoviral BMP-2 Gene Delivery into Canine Bone Marrow-Derived Stem Cells. Orthopedic Research Society Annual Meeting, 2009, San Francisco, CA.

Dewan A K; Calderon N; Dewan R A; Fuentes A; Lazard, Z;; Davis, A; Heggeness, M; **Olmsted-Davis, EA** and Hipp, J. Mechanical Integrity of Spinal Fusion by in situ Endochondral Osteoinduction. Orthopedic Research Society Annual Meeting, 2009, San Francisco, CA.

Z. Lazard, R. Olabisi, L. Hsu, C. Fouletier-Dilling, J. West, F. Gannon, A. Davis, M. Heggeness, **E. Olmsted-Davis**. Non-Invasive Cellular Therapy for Spinal Fusion Treatment. New York Skeletal Society Annual Meeting, 2009, New York, NY.

Merched-Sauvage, M., Kwon, S., Lazard, Z., Salisbury, E, Gannon, F., Davis, A.,Sevick, E, and **Olmsted-Davis, E.** Imaging of heterotopic ossification in advance of bone formation. New York Skeletal Society Annual Meeting, 2009. New York, NY.

E. J. Rodenberg, E. A. Salisbury, Z. W. Lazard, F. H. Gannon, A. R. Davis, and **E. A. Olmsted-Davis.** A Functional Role for Mast Cells in the Production of Brown Adipocytes from Peripheral Nerves in Early Heterotopic Ossification. ASBMR topical meeting, 2009 Bethesda, MD.; Young Investigator Award Winner.

E. Salisbury, Z. Lazard, E. Rodenberg, A.R. Davis, and **E.A. Olmsted-Davis**. Regulation of Early Heterotopic Ossification. ASBMR topical meeting, 2009 Bethesda, MD.; Young Investigator Award Winner.

E. Salisbury, Z. Lazard, E. Rodenberg, A.R. Davis, and **E.A. Olmsted-Davis**. The role of brown adipose in heterotopic ossification. Texas Bone Program Annual Meeting, 2009. Houston TX. Second place winner for best presentation.

Manuscripts:
We have four manuscripts in preparation; however, nothing has been accepted for publication yet.

Below is a description of the outlines for these manuscripts:

1. The first manuscript describes the development of a representative biomechanical test for confirming spine fusion by a relevant clinical standard which is reduction of motion in the lumbar spine.
2. The second manuscript; “An Injectable Method for Spinal Fusion”, describes the cell based gene therapy system, which can reduce mobility, and fuse the spine through a single paraspinous injection. (see appendix)
3. The third manuscript describes the *in vitro* studies with the PEG-DA hydrogel which can selectively be degraded with cathespin K. The manuscript presents all the biochemistry that went into designing the selectively degradable gel, demonstrating with purified cathespin K or general protease can degrade the material but other selective proteases cannot. Finally the manuscript presents the results of the studies to show selective degradation when incubated in the presence of osteoclasts, versus osteoblasts or other types of fibroblasts. The experiments are currently completed and the group is assembled the manuscript.
4. A fourth manuscript is also being assembled to demonstrate the benefits of using the PEG-DA hydrogel microbead structures for bone formation. This manuscript is a comparison study between the microbeads, larger bead structures of the hydrogel, and/or direct injection of the cells without hydrogel. In this study we verify that sustained expression of the BMP2 is optimal for bone formation, and that with inclusion of the hydrogel, we can routinely produce bone at targeted locations, whereas with other types of larger hydrogel structures, we do not obtain any bone formation. We also present some data that demonstrates the length of time the cells are viable in the hydrogel microbead structures, after implantation into the animal, as compared to the cells directly injected, which are rapidly cleared.

Conclusions:

We have demonstrated the ability of BMP2 transdcude cells to induce heterotopic bone formation at a targeted location after a simple injection into the paraspinous musculature. Fusion was similar in either immune compromised or immune competent animals, and appeared to be totally dependent on BMP2 release. The fusion was demonstrated by both radiological and biomechanical analysis, and found to be significantly constrained similar to standards required for successful fusion in humans. Further this heterotopic bone fused to the adjacent skeletal bone, even in the absence of decortification or any surgery. We have also found that with reformulation of the hydrogel that direct comparison studies show it to be actually more efficacious than the cells alone which readily fuse the spine. This is most likely due to the the microbead structures of the hydrogel studies, which provide similar levels of BMP2 production, but significantly extend cell viability at the site of bone formation. We have also completed the studies that demonstrate *in vitro* the selective degradation of the hydrogels by cathespin K or osteoclasts. We have introduced RGD binding sites, to provide cellular binding sites for the cells. We have also made significant progress on defining what cells are actually responding to the BMP2, as well as showing the location in real time of the delivery cells during bone formation. From these preliminary results it appears that the microbead structures keep the cells localized to the injection site and do not circulate. These are the first steps in developing a safe and efficacious noninvasive gene therapy for the tissue engineering of bone.

However, for this system to become a reality, we must determine the minimal number of transduced cells required for spine fusion when delivered through this targeted method. **Here we demonstrate the ability of this injectable material to adequately fuse the spine ≥ 90% success rate.** We must also show similar efficacy in the spines, using the degradable PEG-DA hydrogel microbeads which can be formed ex vivo, and injected directly into the paraspinous musculature. We will also start to implement the human mesenchymal stem cells MSCs, into the degradable hydrogel, so that we can time the viability of the cells to match the remodeling of the material. To do this we will introduce a caspase into the cells that will allow us the ability to induce apoptosis at specific times, just prior to the remodeling of the microbead structures. We propose in this second phase of the grant, to optimize the degradable microbeads, along with the MSCs carrying a caspase gene, to demonstrate the efficacy of the final system. With completion of these final studies and demonstration of spine fusion from injection of a single material we will be poised to initiate safety and toxicology studies for this material.

Completion of this project would significantly advance the current state of gene therapy in this field by eliminating the search for an optimal osteoprogenitor cell and scaffolding. But even more importantly, it would offer a non-invasive alternative to current treatments for degenerative spine disorders. Posterolateral spine fusion, which normally results in 500-1000 cc of blood loss as well as a 5 to 7 day hospital stay and a recovery

period of up to a year, could be performed on an outpatient basis with this minimally invasive procedure, without concern over undue morbidity. This technology would benefit a broad age range of patients, and greatly reduce treatment costs as well as loss work time. Our proposed method has the potential to improve the safety efficacy and recovery times of current spine surgery techniques and would offer an alterative to patients who require spine fusion but are not candidates for major surgery.

References:

1. Hipp JA, Wharton ND. Quantitative motion analysis (QMA) of the spine. In: Yue JJ, Bertagnoli R, McAfee PC et al., eds. *Motion Preservation Surgery of the Spine*. 1st ed. New York: Elsevier Health, 2008

2. Zhao KD, Yang C, Zhao C et al. Assessment of noninvasive intervertebral motion measurements in the lumbar spine. *J.Biomechanics* 2005;38:1943-6.

3. Olmsted-Davis, E. A., Z. Gugala, et al. (2002). "Use of a chimeric adenovirus vector enhances BMP2 production and bone formation." Hum Gene Ther 13(11): 1337-47.

4. Olmsted, E. A., J. S. Blum, et al. (2001). "Adenovirus-mediated BMP2 expression in human bone marrow stromal cells." J Cell Biochem 82(1): 11-21.

5. Thies, R. S., M. Bauduy, et al. (1992). "Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells." Endocrinology 130(3): 1318-24.

6. Fouletier-Dilling, et al (2005). "A Novel Compound Enables High-Level Adenovirus Transduction in the Absence of an Adenovirus-Specific Receptor." Hum Gene Ther 16(11): 1287-1298.

7. Fouletier-Dilling, C. et al (2007). “Efficient and Rapid Osteoinduction in an Immune Competent Host.” Hum Gene Ther 18(8):733-45.

An injectable method for spinal fusion.

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We have achieved spinal fusion via injected BMP2-transduced cells without the use of any carrier, implant, nor decortication of the vertebrae. Spinal fusion surgery remains fraught with varying success rates and complications. Osteoinductive spinal fusion systems hold clinical promise, but present their own challenges and difficulties with inducing targeted rapid bone formation in close proximity to the spinal cord. We previously demonstrated endochondral bone formation in both immunodeficient and immunocompetent mice through the implantation of cells transduced with Ad5F35BMP2. These cells express high levels of bone morphogenetic protein-2 (BMP2), inducing rapid bone formation at targeted sites. Here, we demonstrate the use of this system in achieving consistent and reproducible spinal fusion independent of immune status. Spinal fusion was confirmed through radiographic, microCT, histological and physical examination. Further, the functional performance of the fusion was evaluated with biomechanical testing. These results suggest the ability of our system to induce clinically relevant spinal fusions through a series of injections.

Of the 1 million bone grafts performed worldwide, 50% involve spinal fusions and 25% of these patients complain of donor site pain from the autograft harvest site for up to 2 years post-operatively[2]. The complications associated with autografts have driven the search for and subsequent use of alternative treatments, such as the growing use of bone morphogenetic proteins (BMPs), which have long been demonstrated to induce bone formation[2-5].

Recombinant human BMP-2 (rhBMP-2) is FDA approved on collagen sponges for open long bone fractures and in metal cages for spinal fusion[2]. Without the sponge or the cage, the BMP-2 cannot be localized and tends to diffuse from the desired site, reducing its efficacy and leading to adverse effects such as edema, ectopic bone formation and bone resorption in the graft area[2, 3]. Because BMPs are so rapidly diffused, large quantities of the protein are required, making the procedure very expensive[3]. Furthermore, although the use of rhBMP-2 for spinal fusion may negate the need for an additional operation to harvest autograft bone, these method still necessitates an operation that introduces a permanent foreign object into the body[5]. Furthermore, spinal fusion requires decortication of the transverse processes of the vertebrae targeted for fusion, stripping of all the paraspinous musculature from bone and a fairly long operative time[1, 10]. The decortication and stripping cause pain, and stripping the musculature compromises the stability afforded by these muscles and disrupts the blood supply to both bone and muscle, and promotes scar formation.

Gene therapy approaches have shown promise in combatting the rapid diffusion of BMPs from sponges[2]. These approaches are generally divided into direct gene delivery and cell-based gene delivery methods. Direct gene delivery involves the use of viral or nonviral vectors that encode different osteogenic proteins, such as BMPs,

whereas in cell-based gene delivery cells are first genetically modified *ex vivo* to express a transgene encoding osteogenic proteins and then these altered cells are implanted *in vivo*[2]. These techniques solve the difficulties of maintaining therapeutic BMP-2 levels by using genetically modified cellular machinery to continuously produce the protein. Both direct gene delivery and cell-based gene delivery are highly investigated, but cell-based gene therapy approaches are quickly dominating bone tissue engineering[2]. Cell-based approaches are preferred over direct gene therapy because nonviral approaches are inefficient and viral approaches carry the risk of uncontrolled gene expression, significant autoimmune responses and unexpected side effects[2].

Despite its success at eliminating the need to harvest autograft bone, *ex vivo* gene therapy approaches generally require a carrier for the cells, an operation to implant the cells and involve some decortication of the spine to achieve spinal fusion. As a result, despite the improvements BMP-2 usage has introduced, spinal fusion procedures remain wrought with complications. Therefore, investigations have been driven towards methods for surgery-free spinal fusion.

Previously, through a single injection we achieved endochondral bone formation in immunodeficient and immune-competent murine models with BMP-2 expressing cells[11, 12]. In the immunodeficient model, adenovirus-transduced human diploid fetal lung fibroblasts (MRC-5) were injected into the hindlimb quadriceps muscles of NOD/SCID mice. In the immune-competent model, adenovirus-transduced MC3T3-E1 cells and C57BL/6 mice were used. The MRC-5 cells were transduced *ex vivo* with the adenovirus Ad5F35BMP2; the MC3T3-E1 cells were transduced with Ad5BMP2 and GeneJammer®. To achieve spinal fusion we injected BMP-2 expressing cells along the spine in the paraspinous musculature. Independent of the model, spinal fusion was observed in over 40% of the animals at 2 weeks and in 90% at 6 weeks. In this study, we demonstrate the induction of heterotopic bone in a controlled, targeted manner that consistently fuses to the adjacent vertebral bone in the absence of decortication or injury to this bone. For the first time we demonstrate spinal fusion achieved rapidly, through injection, and without the need for a carrier in both immunocompetent and immunodeficient animals. The rapid nature of the heterotopic bone induction after BMP2 delivery provides for efficient fusion or remodeling with the vertebral bone within 4 weeks of injection. Clinical application of such a technology would provide significant advancement in current treatment options, reducing a risky and complex surgery to a series of injections.

RESULTS

Heterotopic bone formation was observed in all groups receiving cells transduced to express BMP2. In comparison, microCT analyzed of tissues receiving Ad5F35BMP2 transduced cells directly, resulted in the heterotopic bone fusing to one or more lumbar vertebra in 4 of 8 samples (Table 1). Three of these four positive tissues formed bony fusion with solid bony bridging spanning adjacent vertebrae, accomplishing the goal of spinal fusion. Figures 1 and 2 show representative images of fusion in each group.

Table 1: Spinal fusion in injected animals

Group	N	Strain	Time	Spines with 2 vertebrae fused (%)	Spines with 2+ vertebrae fused (%)
Control	9	NOD/ SCID	6 weeks	0 %	0 %
BMP	9	NOD/ SCID	2 weeks	44.4 %	22.2 %
BMP		NOD/ SCID	4 weeks		
BMP	10	NOD/ SCID	6 weeks	90 %	50 %
		C57			

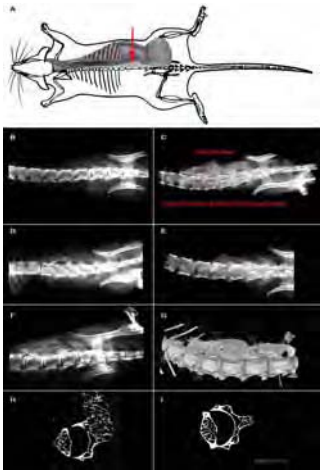

Figure 1: Radiographs show progression of fusion in NOD/SCID mice. A) Injection site. B) Control animal injected with ad5HM4 (hydrogel until I get proper images); C) At 2 weeks fusion is evident from induced scoliosis. D) At 4 weeks remodeling of bone is apparent. E) At 6 weeks bony fusion is remodeled and well integrated into vertebrae. F) C57/BL6 mouse spine at 2 weeks demonstrates efficacy of this system in wild-type mice. G) MicroCT volume rendering of spine in panel B. H) MicroCT slice of fused spine. I) MicroCT slice of normal spine. (Betsy: I do not have the originals of images B, D, F-H.)

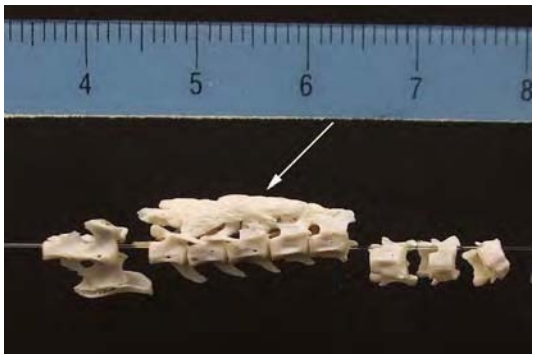

Figure 2: Fused spine suspended on nylon wire. Associated tissue was bleached from the spine and a fishing wire was threaded through the spinal column. Unfused vertebrae hang free, fused vertebrae remain joined and rigid (arrow). Ruler is in millimeters.

Figure 3: Histological evaluation.

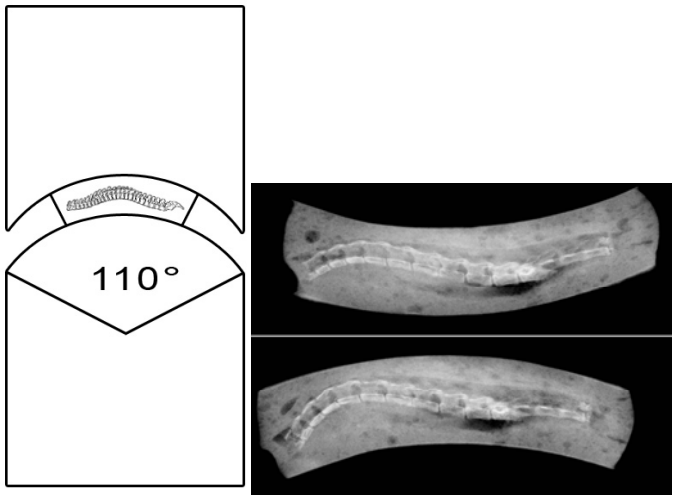

Figure 4: **Left:** Schematic of the in-house flexion device. After 2, 4 or 6 weeks, spines were harvested, placed in the device to induce flexion or extension at 110º and radiographed. **Right:** Representative images used to evaluate biomechanical function of spinal fusion. Top panel shows spine in extension, bottom in flexion. Such images were evaluated with KIMAX QMA software (Medical Metrics, Inc.) to determine whether adjacent vertebrae moved past a predetermined threshold.

DISCUSSION

Our system is the first approach reported in the literature to achieve rapid and clinically relevant spinal fusion in an immunocompetent animal model through injection alone. Furthermore, this system produces bone faster than any other that has been reported and BMP-2 transduction is independent of cell type, enabling its use in any qualified cell line[13]. Previously, we demonstrated that our cell based gene therapy approach was capable of producing rapid heterotopic bone formation in a directed site[11, 12]. The study presented here applies our *ex vivo* approach to spinal fusion. In both the immune compromised (NOD/SCID) and immunocompetent (C57BL/6) models, site specific bone formation led to spinal fusion. This heterotopic bone can directly fuse with vertebral bodies without prior surgical exposure or decortication of the bone. Indeed, in these experiments, some animals developed scoliosis due to the fused spine. Since the epiphyseal growth plates of rodents do not close until two years of age (the approximate life span of laboratory mice), their skeletons essentially do not stop growing[14]. The fusion of the vertebrae in essence mechanically fixed the right side of the spine, causing imbalanced growth, resulting in scoliosis. In addition to confirming spinal fusion by observational methods such as dissection, radiographs, microCT and histological analysis, we demonstrated that these spinal fusions perform functionally through biomechanical analyses of the fused spines. Thus we demonstrate successful spinal fusion in both immunodeficient and immunocompetent animals in a relatively simple system. Other attempts at spine fusion through percutaneous gene therapy approaches are wrought with various difficulties our system avoids.

Many current systems focus on osteoprogenitor cells for spinal fusion; however, these approaches remain largely unsuccessful because of difficulties in obtaining sufficient numbers of mesenchymal stem cells (MSCs) and osteoprogenitor cells[15]. Furthermore, these cells are often delivered with a carrier, such as demineralized bone matrix or synthetic hydroxyapatite[2, 16]. Some strategies employing MSCs genetically alter these cells to emit BMPs, further promoting bone formation; however, an additional characteristic of this approach is that the genetically modified cells form a significant contribution to the newly formed bone[15, 16]. One of the benefits of our system is that the newly formed bone is entirely derived from the host since the adenovirus transduced cells only produce BMP-2 and are not incorporated into the new bone; furthermore, these transduced cells are completely cleared within 7 days of injection regardless of whether the mouse has a complete immune system[12].

Another benefit of our approach is that the use of mesenchymal stem cells is not required, in fact bone formation proceeds independently of cell line used[12]. Nor does our system utilize a carrier to achieve fusion. Reported time scales for spinal fusion using MSCs range between 6 weeks and 3 months; using gene therapy

approaches, spinal fusion typically proceeds between 4 and 12 weeks[2, 15]. Nevertheless, despite the reported successes using these approaches, the successes are tempered with difficulties, whether with MSC populations, poor control over transgene expression or low gene transduction efficiencies[2, 15, 16]. The work presented here circumvents these issues. Bone formation is observed within 1 week of injection, spinal fusion within 2 weeks (in 50% of the animals; 90% at 4 weeks). The rapid onset of bone in addition to the rapid clearance of the transduced cells bodes well for the future therapeutic application of this system in humans. The clinical use of BMPs in human spinal fusion surgery has been largely limited to the surgical implantation of BMPs via traditional open surgical techniques that involve extensive soft tissue dissection and local decortication of the bone. Frequently these procedures are accompanied by surgical bone graft harvest from the pelvis. The creation of a bony fusion by means of the percutaneous injection of a biologically active material, without extensive surgical dissection and bony decortication, would have many clear clinical advantages. The system presented herein could potentially markedly decrease the pain and recovery time for patients undergoing these procedures. Our approach could also drastically reduce blood loss, complication rates, and cost.

METHODS

Cell Culture

Human diploid fetal lung fibroblasts (MRC-5) and murine osteoblasts (MC3T3-E1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and propagated in a humidified incubator at 37°C and 5% CO_2 in α-minimum essential medium (α-MEM; Sigma, St. Louis, MO) and Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 1000 U/L penicillin, 100 mg/L streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen Life Technologies, Gaithersburg, MD), as previously described[12]. Murine stromal cells (W20-17; a gift from Genetics Institute, Cambridge, MA) were propagated and maintained as described by Thies et al.[17].

Adenoviruses

Replication defective E1-E3 deleted first generation human type 5 adenovirus (Ad5) in which the normal fiber protein has been substituted for the human adenovirus type 35 fiber (Ad5F35) were constructed to contain cDNAs for BMP2 in the E1 region of the virus[18]. For the viruses Ad5BMP2, Ad5F35BMP2, Ad5-empty, and Ad5F35HM4 the viral particle (VP)-to-plaque-forming unit (PFU) ratios were 55, 76, 200, and 111, respectively, and all viruses were confirmed to be negative for replication-competent adenovirus. Ad5 viruses were used for the murine MC3T3-E1 cells and Ad5F35 viruses were used for the human MRC-5 cells.

Cell transduction

Because MC3T3 cells lack the coxsackievirus-adenovirus receptor (CAR) for which Ad5 is a ligand, GeneJammer® was utilized in their transduction[11]. MC3T3-E1 cells (1×10^6) were transduced with Ad5BMP2 or Ad5-empty at a viral concentration of 5000 VP/cell with 1.2% GeneJammer®, as previously described[11]. Briefly, GeneJammer® was added at 3% to α-MEM without supplements to and incubated for 10 min at room temperature. Ad5BMP2 or Ad5-empty was then added at the aforementioned concentrations and the mixture was further incubated for 10 min at room temperature. This virus solution was then diluted with supplemented α-MEM to achieve 1.2% GeneJammer® per volume. The resulting solution was next incubated 37°C for 4 hr with cells at an amount that just coated them, then the mixture was diluted with supplemented medium at an amount appropriate for cell culture and incubated at 37°C overnight. MRC-5 cells were transduced as previously described with Ad5F35BMP2 or Ad5F35HM4 at a viral concentration of 2500 VP/cell[19]. Briefly, virus was added to fresh supplemented DMEM and incubated with cells at 37°C overnight.

Animal Care

Female NOD/SCID and C57BL/6 mice (8–12 weeks old; Charles River Laboratories; Wilmington, MA) were placed five per cage and fed with an ad libitum diet and tap water in a 12 h day/night cycle according to Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC) protocols. All animal studies were performed in accordance with standards of the Baylor College of Medicine, Department of Comparative Medicine after review and approval of the protocol by the Institutional Animal Care and Use Committee (IACUC).

Spinal Fusion

Prior to surgery, the back of each mouse was shaved and cleansed with alcohol and then a skin incision was made to reveal the paraspinous muscles. Transduced cells were removed with trypsin, resuspended at a concentration of 5×10^6 cells per 100 μl of PBS, and then delivered by intramuscular injection into the right paraspinous muscles along the length of the spine (Figure #). The skin was then stapled. After 2, 4 and 6 weeks, mice were sacrificed and the spines with attendant musculature were removed and fixed in formaldehyde solution (VWR; Sugar Land; TX).

MicroCT analysis

The intact spines were scanned at 14 µm resolution with a commercial micro-CT system (GE Locus SP, GE Heathcare, London, Ontario). Three-dimensional reconstructions of the spine and any mineralized tissue in the surrounding muscle were created at 29 µm resolution to visualize endochondral mineralized tissues. A volume of interest was defined for each specimen, and a threshold was chosen to exclude any non-mineralized tissue. The total volume of endochondral bone was then measured (eXplore MicroView, v. 2.0, GE Healthcare, London, Ontario) and spines of the animals were not taken into account for the measurements of mineralized tissue in the muscles of the animals.

Histological analysis

Fixed spines were decalcified in EDTA, embedded into a single paraffin block and sectioned at a thickness of 5 µm. The sections were stained with hematoxylin and eosin and observed under light microscopy.

BMP-2 Quantification

BMP-2 expression was evaluated for MC3T3 and MRC-5 cells transduced with Ad5BMP2, Ad5-empty, Ad5F35BMP2 or Ad5F35HM4 using ELISA and alkaline phosphatase assays. Culture supernatant from transduced cells were collected 72 hours after adenovirus transduction and assayed with a BMP-2 Quantikine ELISA kit from R&D Sys-tems (Minneapolis, MN) to measure BMP-2 expression. Transduced cells were cultured in 0.4 µm pore polycarbonate membrane transwell inserts (Corning Inc., Lowell, MA) and W20-17 cells were cultured in the wells of 6 well plates. After 72 hours W20-17 cells were assayed for alkaline phosphatase activity using a chemiluminescence procedure[18]. Three freeze-thaw cycles were performed in a 100-µM/cm^{2}concentration of 25mM Tris-HCl (pH 8.0) and 0.5% Triton X-100 in order to extract cellular alkaline phosphatase and this activity was then measured by adding a ready to use CSPD substrate with Sapphire-II enhancer (Tropix; Applied Biosystems, Foster City, CA) to the samples. After a 2 sec delay, the light output from each sample was integrated for 10 sec with a luminometer (TD-20/20; Turner BioSystems, Sunnyvale, CA). Alkaline phosphatase levels were recorded in relative luminescence units (RLU) and normalized to protein content with the bicinchoninic acid (BCA) assay, using bovine serum albumin to derive a standard curve. Data are presented as percent induction relative to that of unstimulated basal control cells.

Biomechanical Testing

Prior to sectioning, formaldehyde fixed spines were encased in alginate in order to obtain flexion and extension radiographs. Spines were suspended in an in-house mold. Alginate powder was combined in an equal volume to water (30 ml of each) and mixed until smooth. The alginate was poured into the mold and allowed to solidify. Solidified molds were placed in an in-house spring-loaded clamp with rigid 100° arcs (Figure 4). Radiographs were taken of molds in flexion and extension orientations for each spine. These digitized radiographs were used to quantify intervertebral motion with Food and Drug Administration (FDA)-approved software (KIMAX QMA, Medical Metrics, Inc.) that has been validated to measure intervertebral motion with an accuracy of better than 0.5° of rotation and 0.5 mm of translation[20, 21]. Intervertebral motion was measured at each level. Preliminary data (unpublished) demonstrated that the normal mouse spine undergoes an average of 5° of intervertebral motion using this test. Spines were considered fused when adjacent vertebrae did not exhibit rotation beyond 1.5°.

Spine Bleaching

To confirm results obtained from the spinal flexion device, a representative sample of spines (n = ??) were immersed in bleach for approximately one hour after the mechanical tests were completed. This process removes the soft tissues from the vertebral bodies. Midway through this process, nylon wire was threaded up the spinal canal in order to maintain the relative position of the vertebrae.

Statistical Analysis

Statistical analysis was performed as described previously[12]. Briefly, all data were taken in triplicate and reported as mean and standard deviation. A Student t test with 95% confidence interval ($p < 0.05$) was done between the untreated control and each experimental condition.

REFERENCES

1. RA Deyo, A Nachemson and SK Mirza. 2004. Spinal-Fusion Surgery—The Case for Restraint. *The Spine Journal* 4(5S):138-142.

2. N Kimelman, G Pelled, GA Helm, J Huard, EM Schwarz and D Gazit. 2007. Review: Gene-and Stem Cell-Based Therapeutics for Bone Regeneration and Repair. *Tissue Engineering* 13(6):1135-1150.

3. MP Lutolf, FE Weber, HG Schmoekel, JC Schense, T Kohler, R Müller and JA Hubbell. 2003. Repair of bone defects using synthetic mimetics of collagenous extracellular matrices. *Nature Biotechnology* 21:513-518.

4. Y Liu, EB Hunziker, CV Vaal and K Groot. 2004. Biomimetic Coatings vs. Collagen Sponges as a Carrier for BMP-2: A Comparison of the Osteogenic Responses Triggered in vivo Using an Ectopic Rat Model. *Key Engineering Materials* 254:619-622.

5. F Mussano, G Ciccone, M Ceccarelli, I Baldi and F Bassi. 2007. Bone Morphogenetic Proteins and Bone Defects: A Systematic Review. *Spine* 32(7):824.

6. HG Schmoekel, FE Weber, JC Schense, KW Graetz, P Schawalder and JA Hubbell. 2005. Bone repair with a form of BMP-2 engineered for incorporation into fibrin cell ingrowth matrices. *Biotechnology and Bioengineering* 89(3):253-262.

7. FE Weber, G Eyrich, KW Grätz, FE Maly and HF Sailer. 2002. Slow and continuous application of human recombinant bone morphogenetic protein via biodegradable poly (lactide-co-glycolide) foamspheres. *International Journal of Oral & Maxillofacial Surgery* 31(1):60-65.

8. L Madigan, AR Vaccaro, MR Lim and JY Lee. 2007. Bioabsorbable Interbody Spacers. *Journal of the American Academy of Orthopaedic Surgeons* 15(5):274.

9. BP Hecht, JS Fischgrund, HN Herkowitz, L Penman, JM Toth and A Shirkhoda. 1999. The Use of Recombinant Human Bone Morphogenetic Protein 2 (rhBMP-2) to Promote Spinal Fusion in a Nonhuman Primate Anterior Interbody Fusion Model. *Spine* 24(7):629.

10. M Bikram, C Fouletier-Dilling, JA Hipp, F Gannon, AR Davis, EA Olmsted-Davis and JL West. 2007. Endochondral Bone Formation from Hydrogel Carriers Loaded with BMP2-transduced Cells. *Annals of Biomedical Engineering* 35(5):796-807.

11. CM Fouletier-Dilling, P Bosch, AR Davis, JA Shafer, SL Stice, Z Gugala, FH Gannon and EA Olmsted-Davis. 2005. Novel compound enables high-level adenovirus transduction in the absence of an adenovirus-specific receptor. *Human Gene Therapy* 16(11):1287-1297.

12. CM Fouletier-Dilling, FH Gannon, EA Olmsted-Davis, Z Lazard, MH Heggeness, JA Shafer, JA Hipp and AR Davis. 2007. Efficient and Rapid Osteoinduction in an Immune-Competent Host. *Human Gene Therapy* 18(8):733-745.

13. Z Gugala, EA Olmsted-Davis, FH Gannon, RW Lindsey and AR Davis. 2003. Osteoinduction by ex vivo adenovirus-mediated BMP2 delivery is independent of cell type. *Gene Therapy* 10(16):1289-1296.

14. SH Windahl, G Andersson and JÅ Gustafsson. 2002. Elucidation of estrogen receptor function in bone with the use of mouse models. *Trends in Endocrinology & Metabolism* 13(5):195-200.

15. ON Gottfried and AT Dailey. 2008. Mesenchymal Stem Cell and Gene Therapies for Spinal Fusion. Topic Review. *Neurosurgery* 63(3):380-392.

16. H Aslan, D Sheyn and D Gazit. 2009. Genetically engineered mesenchymal stem cells: applications in spine therapy. *Regen. Med.* 4(1):99-108.

17. RS Thies. 1992. Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. *Endocrinology* 130(3):1318-1324.

18. EA Olmsted, JS Blum, D Rill, P Yotnda, Z Gugala, RW Lindsey and AR Davis. 2001. Adenovirus-

Mediated BMP2 Expression in Human Bone Marrow Stromal Cells. *Journal of Cellular Biochemistry* 82(1):11-21.

19. AR Davis, NA Wivel, JL Palladino, L Tao and JM Wilson. 2001. Construction of adenoviral vectors. *Molecular biotechnology* 18(1):63-70.
20. JA Hipp, CA Reitman and N Wharton. 2005. Defining Pseudoarthrosis in the Cervical Spine With Differing Motion Thresholds. *Spine* 30(2):209.
21. K Zhao, C Yang, C Zhao and KN An. 2005. Assessment of non-invasive intervertebral motion measurements in the lumbar spine. *Journal of biomechanics* 38(9):1943-1946.